

**Immune responses during jaagsiekte sheep retrovirus infection
and development of ovine pulmonary adenocarcinoma**

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Declaration

I hereby declare that:

- a) this thesis has been composed by myself
- b) it has not been submitted for candidature to any other degree
- c) the work has been performed by myself. Some of the immunohistochemistry procedures were performed in collaboration with colleagues at Moredun Research Institute. Where such work was carried out, this fact is acknowledged.

Christina Summers

April 2002

**I dedicate this thesis to the memory of my Grandfather,
Mr. William Blakie Downie
who first awakened my interest in science**

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ABSTRACT

Jaagsiekte sheep retrovirus (JSRV) is the aetiological agent of ovine pulmonary adenocarcinoma (OPA), a contagious bronchioloalveolar carcinoma, which imposes a serious economic burden on the sheep farming industry. This study evaluated the immunological responses during experimentally induced JSRV infection and tumorigenesis in conventionally housed and specific pathogen free (SPF) neonatal lambs, and in adult field cases in the terminal stages of OPA.

This study identified, for the first time, changes in cellular function in the peripheral blood of JSRV-infected animals by measuring the *in vitro* lymphoproliferative response to various mitogens. The presence of JSRV did not affect the level of response to phytohaemagglutinin (PHA) and pokeweed mitogen (PWM) stimulation, but a reduced response to concanavalin A (ConA) was demonstrated in the JSRV-infected animals. The reduced response to ConA was detected prior to the diagnosis of clinical symptoms and was also evident in the terminal stages of OPA. The presence of JSRV also affected the mitogenicity of the mannose-specific, monocotyledonous *Narcissus pseudonarcissus* lectin (NPA). The level of proliferation was comparable between JSRV-infected and control lambs, but post-stimulation phenotyping revealed an altered phenotypic profile, with elevated numbers of T and B lymphocytes from JSRV-infected animals. Furthermore, the addition of exogenous mannose completely inhibited NPA mitogenicity in control but not in JSRV infected lambs.

It has been established that NPA possesses insecticidal properties, which could potentially increase pest resistance in transgenic crops. During this study we revealed that NPA mitogenicity is age-dependent in sheep, with no lymphoproliferative response detected in adult animals. This research was extended to include human subjects, and we have shown that NPA is slightly mitogenic for adult lymphocytes but that mitogenicity is increased more than sevenfold for lymphocytes purified from umbilical cord blood. These findings indicate possible physiological implications as a result of introducing foreign lectins into human and animal food sources.

The peripheral blood mononuclear cells (PBMC) phenotypic profile was monitored during acute experimental JSRV infection, and also the immune status of natural OPA cases, in the terminal stage of the disease, was assessed. The PBMC were labeled using a panel of mouse monoclonal antibodies recognising sheep PBMC subsets, and results determined by flow cytometry. Neutrophils were counted also. The results confirmed the CD4 lymphocytopenia and neutrophilia previously demonstrated in the blood of terminal OPA field cases. In contrast, no PBMC subset frequency alterations were detected, at any time in JSRV-infected lambs, which could have indicated an immunological response to JSRV infection. Moreover, the results showed that lymphocytopenia and neutrophilia do not occur during experimentally induced infection leading to OPA. Hence, they are not an early event of tumorigenesis and may not be in direct response to JSRV infection but a consequence of secondary infections encountered during declining health.

At necropsy, samples of lung and tumour were collected from JSRV-infected and control SPF lambs for immunohistochemical analysis. A novel aldehyde-free, zinc salts fixative (ZSF) originally produced for immunolabeling human samples was adapted and developed for ovine tissues. Antigen-retrieval steps were omitted with ZSF and immunolabeling of otherwise fixation-sensitive antigens was possible. This method also preserved the cellular morphology and delicate architecture of the lung tissue. No difference in the distribution of immune cells was identified in the non-affected area of the OPA lung when compared with the uninfected lung. Immunolabeling tumour samples revealed unexpectedly that very few tumour infiltrating lymphocytes were present, located to the periphery of the tumour nodules.

ABBREVIATIONS

| | |
|---------------------|--|
| α | alpha |
| ADCC..... | antibody dependent cell cytotoxicity |
| β | beta |
| β -gal..... | betagalactosidase |
| BAL..... | bronchiolalveolar lavage |
| BALT..... | bronchial associated lymphoid tissue |
| BSA..... | bovine serum albumin |
| Ci..... | curie |
| CFSE..... | carboxyfluorescein diacetate succinimdy ester |
| Con A..... | concanavalin A |
| CTL..... | cytotoxic T lymphocytes |
| DC..... | dendritic cells |
| DNA..... | deoxyribonucleic acid |
| <i>E.coli</i> | <i>Escherichia coli</i> |
| ELISA..... | enzyme-linked immunosorbent assay |
| ER..... | endoplasmic reticulum |
| FDC..... | follicular dendritic cells |
| γ | gamma |
| GM-CSF..... | granulocyte macrophage-colony stimulating factor |
| GST..... | glutathione-S transferase |
| HBSS..... | Hanks balanced salt solution |
| IFN- α | interferon alpha |
| IFN- β | interferon beta |
| IFN- γ | interferon gamma |
| Ig..... | immunoglobulin |
| IHC..... | Immunohistochemistry |
| IL..... | Interleukin |
| JSRV..... | jaagsiekte sheep retrovirus |
| kb..... | kilobase pairs |
| kDa..... | kilodaltons |
| MAb..... | monoclonal antibody |
| MBq..... | Mega-Becquerel |
| mg..... | milligram (10^{-3} gram) |
| MHC..... | major histocompatibility complex |
| mins..... | minute(s) |
| ml..... | millilitre (10^{-3} litre) |
| MRI..... | Moredun Research Institute |
| M.wt..... | molecular weight |
| NaCl..... | sodium chloride |
| NK cells..... | natural killer cells |
| NPA..... | <i>Narcissus pseudonarcissus</i> agglutinin |
| OPA..... | ovine pulmonary adenomatosis |
| ORF..... | open reading frame |
| PBMC..... | peripheral blood mononuclear cells |
| PBS..... | phosphate buffered saline |

| | |
|----------------------|--|
| PCR..... | polymerase chain reaction |
| PHA..... | phytohaemagglutinin |
| PWM..... | pokeweed mitogen |
| RNA:-..... | ribonucleic acid |
| mRNA..... | messenger ribonucleic acid |
| RPMI..... | Rosewell Park Memorial Institute |
| STWS..... | Scotts tap water solution |
| SPF..... | specific pathogen free |
| TAP..... | transporter associated with antigen presentation |
| TGF..... | Tumour growth factor |
| T _H | T helper cells |
| TNF- α | tumour necrosis factor alpha |
| TNF- β | tumour necrosis factor beta |
| μ Ci..... | microcurie |
| μ g..... | microgram |
| μ l..... | microlitre |
| μ m..... | micrometer |
| v/v..... | volume to volume |
| wt/v..... | weight to volume |
| ZFS..... | zinc salts fixative |

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Chapter 1

Introduction

1.1 Historical origins of ovine pulmonary adenocarcinoma

Contagious bronchioloalveolar cancer now known as ovine pulmonary adenocarcinoma (OPA) is not a recently described disease. It's origins can be traced as far back as the nineteenth Century in South Africa, where it has been suggested that the disease was introduced by the importation of Spanish Merino sheep (Tustin 1969, Wandera 1970). In the sheep farming regions of South Africa, harsh climatic and environmental conditions forced sheep farmers to live a semi-nomadic lifestyle, driving their flocks from one area to another, often over long distances, to provide adequate grazing for their animals. It was at the end of the sheep droves that shepherds began to detect clinical symptoms such as severe weight loss and laboured breathing. These symptoms were not restricted to a few isolated animals but were extensive enough to be regarded as a recognised disorder. The condition became known as driving sickness, Jaagsiekte, an Afrikaans term, 'jaagt' meaning driving or droving and 'siekte', a corruption of the word ziekte meaning sickness. The term jaagsiekte was first used in a scientific context by Robertson (1904) and even today jaagsiekte is still a recognised alternative nomenclature for the disease also previously termed ovine pulmonary carcinoma (OPC) or sheep pulmonary adenomatosis (SPA) and recently renamed ovine pulmonary adenocarcinoma (OPA).

1.2 Epidemiology

Epidemiological studies of transmissible diseases in domesticated animals are an onerous undertaking as many parameters must be considered that affect the incidence. Sheep management policies vary considerably not only on a global scale but also at a regional level. For example the methods of farming lowland sheep

where considerable contact with the animals is maintained, are quite different from the relative isolation of hill sheep. Other factors include over-wintering conditions, breeding programmes, veterinary care and sheep management policies.

The variations are aptly demonstrated when analysing the 1934 – 1952 cases of OPA in Iceland, where the mortality rate ranged from 50-80% of sheep in some affected flocks. This outbreak was first identified in 1933, following the importation of rams from Germany, (Dungal *et al* 1938). Importation of sheep in the eighteenth and early nineteenth Centuries did not result in any OPA cases. Therefore the introduction of the Karakul breed in 1933 was regarded as the initial reservoir of infection (Tustin 1969, Palsson 1985). The mode of sheep management in Iceland has been strongly implicated in the spread of infection (Dungal 1938, Tustin 1969, Palsson 1985). On the mountainside, during the summer months, flocks from several farms intermingle freely. In autumn, flocks that may contain up to 4,000 sheep, are herded into large holding pens where they are separated for ownership by earmark; a process, which may take up to 3 days to complete. The individual flocks are then over-wintered in large sheep sheds for up to 5 months, depending on weather conditions. The unique nature of sheep farming and flock management in Iceland is ideally suited to the spread of infection (Verwoerd 1985). The subsequent eradication of OPA from Icelandic flocks was achieved only by severe culling. To date no recurrence of OPA has been reported in Icelandic flocks.

1.2.1 Global distribution of OPA

In Australia and New Zealand, only two cases of OPA have been reported despite the intensity of sheep farming, but OPA has been detected with varying prevalence in many major sheep farming regions worldwide. The primary source for

the epidemiological evaluation of OPA incidence was from the study of tissue samples obtained from abattoirs. The recorded incidence of OPA (based on such studies) was variable, for example, 0.08% in Spain (Dualde-Perez 1963), 0.1% in Peru (Cuba Caparo *et al* 1961), 0.2% in Northern Germany (Schultz *et al* 1965), 1.6-7.5% in Russia (Aliev 1967), 10-30% in Greece (Christodoulous and Tarlatzis 1952), and with up to 37% recorded in France (Moraillon and Yalcin 1967). The first recorded incidence of OPA in Malaysia was identified in purebred, imported sheep and crossbreeds in 1993 (Zamri-Saad *et al* 1995). Within a year an outbreak of OPA resulted, and subsequently more cases have been reported throughout the country (Krishnan and Paul 1994, Azman Shah *et al* 1996).

OPA imposes a serious economic burden on the sheep farming industry due to the high mortality rates in infected flocks. During the Bulgarian epidemic, a mortality of 36% was recorded for some farms (Enchev 1961). In Greece between 10% and 30% losses were documented (Christodoulous and Tarlatzis 1952), and up to 30% losses annually were calculated for some Kenyan (Shirlaw 1959) and South African farms (Tusin 1969). Regrettably the financial implications are not the only consequence of OPA. In 1987 Nieddu *et al* reported the presence of OPA in a flock of over 60 moufflon (*Ovis munsimon*) owned by the Wildlife Department of the Sardinian Government. With wild moufflon now designated as an endangered species, the purpose of rearing this flock was to return animals to the wild. Unfortunately measures to control the spread of infection were not employed early enough, resulting in spread to the indigenous wild population.

1.2.2 Epidemiology of OPA in Britain

In the UK, a case of verminous pneumonia (Dykes and McFadyean 1888) retrospectively turned out to be the first description of OPA in Britain. This report, together with findings recorded by McFadyean (1920), were re-examined as a result of reports from South Africa (Robertson 1904, Cowdry and March 1927, de Kock 1929) and it was concluded that verminous pneumonia was a misdiagnosis of OPA.

The first major outbreak of OPA in Britain was reported in 1941 by Blakemore and Bosworth. It was located in Norfolk and implicated Scottish crossbreeds as the reservoir for infection. However, the first outbreak of OPA to actually occur in Scotland was not reported until 1946 by Harbour and Jamieson. The breeds implicated in this incidence were Scottish Blackface and half-breeds from Aberdeenshire and Berwickshire. A second outbreak in England, again in Norfolk, was reported by Stevens (1957). On this occasion the Scottish Greyface breed was involved. The significance of Scottish sheep in OPA outbreaks continued, from the observations by Mackay and Nisbet (1966) to the most recent British epidemiological surveys, 1975 – 1981, recorded by Hunter and Munro (1983).

Mortality, as a result of OPA, of less than 1% in British flocks was estimated by MacKay and Nisbet in 1966, but the mortality rate within affected flocks is now thought to be much higher. Studies of three flocks, over the period 1980 – 1981 (Hunter and Munro 1983) and two flocks, studied over a five-year period, suggest a 3% mortality (Sharp 1981). However, findings of the 1975 – 1979 and 1980 – 1981 Scottish surveys (Hunter and Munro 1983) report that 20% of all adult sheep, examined at post mortem, had OPA lesions; comparative studies undertaken by Williams and Ross (1983) estimate this figure at 21%. These data may be grossly

underestimated as only a very small proportion of the sheep population undergo necropsy examination. Therefore the precise prevalence of OPA has not been determined. This emphasises the necessity for a reliable diagnostic test to identify OPA in the field, which would allow accurate, epidemiological surveillance.

1.2.3 Breed and sex of animals affected by OPA

Confirmed OPA cases have been detected in many breeds of sheep, throughout the world. However, there are conflicting opinions regarding the susceptibility to OPA in various breeds, with reports suggesting that the Icelandic Gottorp (Dungal *et al* 1938) and some British breeds such as Blackface, Greyface, Cheviot and Half-breed (Martin *et al* 1979, Hunter and Munro 1983) are highly susceptible, whilst other breeds are more resistant. There are many factors to consider in determining breed susceptibility, as the geographical location, climatic and environmental conditions are influential in determining the mode of husbandry and sheep management policies. A specific breed, indigenous or selected for its suitability to a particular mode of farming, is frequently the predominant breed within a region. Hence all the external factors pertaining to sheep farming within a region must be considered and comparisons made with other breeds farmed under the same conditions. It would be extremely difficult therefore to compare the susceptibility to OPA in breeds that are managed under very different conditions.

Reports into gender susceptibility for OPA are conflicting, with some indicating an equal level of incidence in males and females (Damodaran 1960, Tusin 1969) and others suggesting that males are more susceptible than females (De Kock 1929, Kostenko 1968). As with breed susceptibility, external factors in the management of ewes and rams must be considered. It was observed by Mackay and

Nisbet in the 1966 survey, that rams and ewes are farmed in completely different ways. Rams are frequently intensely managed, housed together and receive more veterinary care than ewes. Furthermore, rams generally tend to be kept longer than ewes and therefore exposure to possible infection is increased, and also the time to develop OPA.

1.3 The aetiology of OPA

In a report from 1929, De Kock voiced his suspicions that OPA was a transmissible disease, but perhaps the first descriptions of a possible aetiological candidate preceded this when Robertson in 1904, described protozoan-like crescent-shaped bodies in OPA lesions. Mitchell (1915) however, discounted this theory, as his examination of OPA lesions did not detect any protozoa. Notwithstanding, the parasite theory was still upheld by McFadyean in 1920. Mycoplasmas as causative agents were examined (Mackay *et al* 1963) but rejected when it was found that they were not present in all OPA cases (Wandera 1971) and furthermore, that identical mycoplasmas to those identified in OPA were discovered in association with maedi visna, *Pasteurella pneumonia* and were also frequently isolated from the lungs of uninfected, healthy sheep (Mackay and Nisbet 1966, Wandera 1968, 1971, Jones 1978).

1.3.1 Evidence implicating a viral infection

For many decades the aetiological agent of OPA remained elusive, but viral particles were detected in OPA tumours by Perk *et al* (1971), providing the first indication that a virus might be implicated. Morphologically the viral particles were characteristically retroviral, with the detection of type A particles in the epithelial cells. Furthermore, type C particles were described budding from the stromal cells in

the tumour nodule (the detection of type A and C viral particles was subsequently not substantiated). Studies of tumour extracts were extended to further characterise the biochemical and morphological features of the virus (Perk *et al* 1974, Martin *et al* 1976). Viral particles with a density of 1.15-1.20gm/ml were identified, containing a reverse transcriptase, further substantiating the characteristics of a retrovirus. The reverse transcriptase activity in OPA tumour and lung fluid was further characterised (Verwoerd *et al* 1980, Herring *et al* 1983) and the virus was named Jaagsiekte sheep retrovirus (JSRV), the taxonomy reflecting the Afrikaans origins of the disorder (York *et al* 1991).

1.3.2 Retrovirus taxonomy and classification

The person who discovered the virus traditionally has the privilege to name it, with the host species and possibly the disease associated to infection often included in the name. The addition of a persons name in viral nomenclature is no longer acceptable, although several viruses, which were identified prior to this convention, do exist such as Rous sarcoma virus. The International Committee on the Taxonomy of Viruses is responsible for the classification of all viruses. The family *Retroviridae* is subdivided into seven subfamilies at present: mammalian C-type retroviruses, the prototype being murine leukaemia virus (MLV); avian C-type, with the prototype Rous sarcoma virus (RSV); the B-type retroviruses, prototype mouse mammary tumour virus (MMTV); D-type, with the prototype Mason Pfizer monkey virus (M-PMV); lentiviruses, with human immunodeficiency virus (HIV) as the prototype; the human T-cell leukaemia virus (HTLV)/bovine leukaemia virus (BLV) group, with the prototype HTLV-1 and the spumaviruses represented by the prototype human foamy virus (HFV). The classification of each genera is based on several criteria

including sequence similarities in their reverse transcriptase, viral core morphology and the location within the cell (cytoplasm or plasma membrane) of core assembly, and whether the virus possesses additional accessory genes.

1.3.3 Retrovirus structure and life cycle

The retrovirus structure is very simple, composed of a core containing the viral genomic material encapsulated within an envelope, which is enriched with viral glycoproteins. Contained within the viral core, the genome comprises two identical single strands of RNA and the enzymes necessary for retroviral replication, reverse transcriptase, integrase and protease. When integrated into the host genome the provirus comprises two long terminal repeat (LTR) sequences, which encode many regulatory signals and flank the open reading frames (ORF). The *gag* gene encodes the core proteins, the *pol* gene encodes for the enzymes reverse transcriptase, integrase and protease and the *env* gene encodes the envelope glycoproteins, which modify the envelope that has been constructed from the membrane of the previous host. Some retroviruses also possess oncogenes or proteins with specialized regulatory functions.

Retroviral entry into the cell is mediated by recognition and interaction of the viral envelope proteins with specific cell surface receptors (Figure 1.1). The surface protein (SU) binds to the receptor, which brings the viral and cell membranes into close contact, resulting in a conformational change that allows the viral transmembrane protein (TM) to mediate membrane fusion. Some retroviruses enter the cell by receptor-mediated endocytosis (RME); the exploitation of a mechanism probably evolved by the cell for intracellular ingestion of beneficial extracellular

products. After entry into the cell reverse transcription of the retroviral RNA and integration of the proviral DNA into the host genome follows.

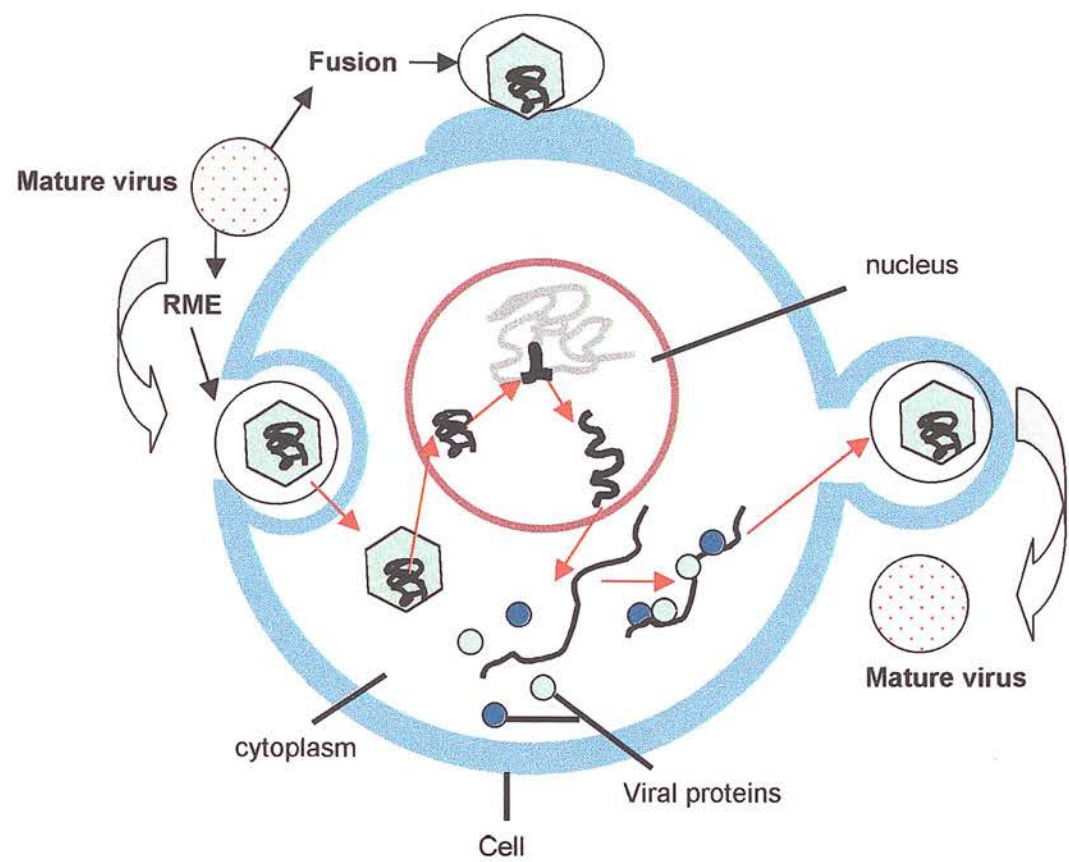


Figure 1.1 Retrovirus life cycle. A mature virus particle enters the cell by receptor mediated endocytosis (RME) or fusion, uncoats to form a nucleoprotein complex, which mediates viral RNA reverse transcription and integration of the provirus (black) into the host chromosomal DNA (grey). Transcription of proviral DNA and translation of viral RNA. The proteins in the cytoplasm are combined with the RNA and associated to the viral envelope, resulting in assembly of a mature viral particle, which then buds out of the cell.

The use of the cell RNA polymerase II in the transcription of the provirus, the mechanism used to cap, polyadenylate and splice the host RNAs, is exploited by the virus for processing RNA transcripts. The virus also utilizes the host polyribosomes for the translation of retroviral RNA. To complete viral replication recognition signals, which are not fully understood as yet, induce the viral RNA and core

proteins from the cytoplasm to associate with the envelope glycoproteins implanted in the plasma membranes. Finally, the viral encoded protease cleaves the polyproteins to generate the smaller polypeptides identified in mature retrovirus and the viral particles exit the cell by budding from the plasma membrane.

1.3.4 Retroviral induced oncogenesis

Although the majority of retroviral infections are non-pathogenic, several oncogenic retroviruses have been isolated from species of vertebrates including fish, chickens, rodents, cats, primates and humans. Epithelial carcinomas, sarcomas and various types of leukaemias have been identified (Weiss *reviewed* 1982, 1985). Oncogenic retroviruses are categorised into two groups, (i) retroviruses such as Rous sarcoma virus (RSV), which possess a viral oncogene responsible for inducing tumours and the ability to transform cells in culture (Bishop and Varmus 1982). The oncogene, integrated into the host genome is under control of the viral LTR, promoters and enhancers, which results in over expression of the protein and induces transformation. (ii) retroviruses such as mouse mammary tumour virus (MMTV) lack oncogenes but can control the host genes by proviral insertion in a position adjacent or near to a cellular oncogene. The viral LTR overrides the host control and places the gene under the control of the virus. Therefore transformation of the host genome can occur either by transduction or insertional activation.

1.4 Detection of viral antigens by the immune system

To evoke an immune response the immune system must detect the presence of foreign antigens and then deploy strategies to combat the infection. These responses can be categorized into innate immunity, which is non-specific and utilizes

interferon, natural killer (NK) cells and phagocytes and adaptive immunity, which is highly specific for a particular pathogen and involves the recognition of antigen by specific T and B lymphocytes.

1.4.1 Innate immune responses to viral infection

Should virus survive past the outer defences such as IgA at mucosal surfaces, which inhibits virus adsorption and penetration, the first line of defence encountered is the innate immune system. As innate immunity is rapidly activated, within minutes or only a few hours after viral infection, it serves to protect the host by restricting early infection and the spread of virus. Against viral infection one of the first mechanisms in innate defence is the production of interferon (IFN). IFN is categorized into two types; type I, which includes IFN- α and β and type II which includes IFN- γ . Within hours of viral infection, interferon is induced which activates the surrounding cells, *via* their IFN- α/β receptors, to synthesize more IFN (Vilcek and Sen 1996). The anti-viral mechanism used by IFN is determined by the type of virus, cell and interferon induced. Several interferon strategies to inhibit viral infection have been demonstrated. For example the disruption of viral replication by inhibition of protein synthesis is the mechanism used against adenovirus, influenza viruses and vaccinia virus (Hovanessian 1989), or the degradation of viral RNA, as demonstrated for picornaviruses (Sen and Ramsohoff 1992). It has also been shown that interferon can disrupt the penetration and uncoating of some retroviruses. In addition to direct anti-viral activity interferon is also implicated in the activation of NK cells, the up regulation of MHC, proteosomes and the TAP proteins and is associated with the terminal differentiation of T and B lymphocytes. Herpesviruses have been found particularly susceptible to the activity of NK cells (Shanley 1990).

The large granular NK cells are characterized by their ability to kill certain tumour cell lines *in vitro*. They are distinct from the T and B lymphocyte lineage but can produce many of the anti-viral cytokines such as IFN- γ , tumour necrosis factor α (TNF- α), tumour growth factor β 1 (TGF- β 1), granulocyte/macrophage-colony stimulating factor (GM-CSF) and interleukin-1 β (IL-1 β) (Trinchieri 1989, Perussia 1991). The mechanisms by which NK cells recognize virus infected cells is unknown but an inverse correlation between the level of MHC class I expression and killing by NK cells has been demonstrated (Liao *et al* 1991). It has been established that NK cells can be activated to participate in antibody-dependent cell cytotoxicity (ADCC), the recognition of IgG antibody-coated cell surfaces, resulting in the subsequent killing of the target cell (Kagi *et al* 1994).

Macrophages and neutrophils can also kill virus-infected cells by ADCC through the opsonization of virus-antibody complexes or by direct intracellular killing *via* phagocytosis of the infected cell. The majority of IFN- α detected in the blood following infection is produced by the macrophages. In addition macrophages produce TNF- α , which can destroy virus-infected cells by apoptosis (Wong and Goeddel 1986), and arginase, which depletes the concentration of arginine at the local site (Bonina *et al* 1984). Furthermore, following macrophage activation by IFN- γ nitric oxide synthase production is induced that results in the generation of nitric oxide, which inhibits vaccinia virus and HSV replication (Karupiah *et al* 1993).

1.4.2 Adaptive immune responses to viral infection

Whilst transcriptionally inactive the retrovirus remains invisible to the immune system. Once a retrovirus is active within a cell the potential to elicit an immune response to viral antigens becomes evident. Retroviral infection is

predominantly cell-associated therefore it is the recognition and destruction of infected cells rather than free viral particles that is central in combating infection. Consequently, the cell-mediated response by cytotoxic T lymphocytes (CTL) predominates, although specific antibody responses can also be induced. The production of anti-viral antibodies confers an obvious advantage to the host, not only in directly controlling the spread of infection but also in contributing to the destruction of virus-infected cells.

1.4.2.1 The role of antibodies in viral infection

The first antibody identified responding to viral infection is IgM, which despite generally having low affinity does control the spread of virus in the blood. As the humoral response progresses and immunoglobulin class switching occurs, IgG with a high affinity for viral antigens predominates in the serum (Wabl and Steinberg 1996), whilst the principal antibody identified at mucosal surfaces is IgA. The mechanism by which neutralization of virus occurs is dependant not only on the viral epitope and the class of antibody but also the nature of the infected cell (Dimmock 1995). Neutralization can occur at the cell surface, for example by disrupting fusion of the viral envelope and the cellular membrane (Wharton *et al* 1995), or intracellularly by blocking uncoating of the virus (Vrijzen *et al* 1993). The involvement of antibodies in the destruction of virus-infected cells has also been demonstrated, for example in the ADCC process described in section 1.4.1.

1.4.2.2 Presentation of viral antigens by MHC class I molecules

To be recognised by the adaptive immune system, the viral antigen must be presented, in conjunction with major histocompatibility complex (MHC). MHC restriction determines the type of T cell response elicited. All nucleated cells express

MHC class I that is recognised by cytotoxic T lymphocyte (CTLs), therefore the immune system has the opportunity to identify endogenous viral antigens presented on the surface of virtually all cells. Processing and presentation of virus *via* the endogenous antigen-presenting pathway is a multi-step process (Figure 1.2) starting with the degradation of viral proteins located in the cytoplasm into small peptides by the proteasome, a complex of proteolytic enzymes. The peptides are then transported to the endoplasmic reticulum (ER) by the transporter associated with antigen presentation (TAP) proteins. Two MHC-encoded subunits TAP1 - 2 complex to form a heterodimer. In the ER the peptides bind to the MHC class I heavy chain. As the MHC binding cleft will accommodate peptides of eight - fifteen amino acids the peptide is further trimmed if required. With the assistance of molecular chaperones including calnexin, calreticulum and tapasin, the MHC light chain (β -microglobulin) associates with the heavy chain to form the MHC class I trimolecular complex. This is now transported through the ER and the Golgi apparatus to the plasma membrane where it is recognised by the CTLs, which kill the infected cell.

1.4.2.3 Presentation of viral antigens by MHC class II molecules

Exogenous viral peptides are processed and presented complexed to MHC class II molecules that are expressed by antigen presenting cells (APC) and B lymphocytes. The MHC class II-antigen complex is recognized by helper T (T_H) cells. MHC class II, composed of an α and β chain is located in the ER, complexed to a polypeptide, the invariant chain (I_i), which is encoded by a gene out with the MHC. Unlike MHC class I, the class II $\alpha\beta$ -I_i complex does not encounter antigen in the ER and is not transported directly to the cell surface, but is transported through the Golgi apparatus to an acidic endosomal or lysosomal compartment where the I_i

MHC class 1

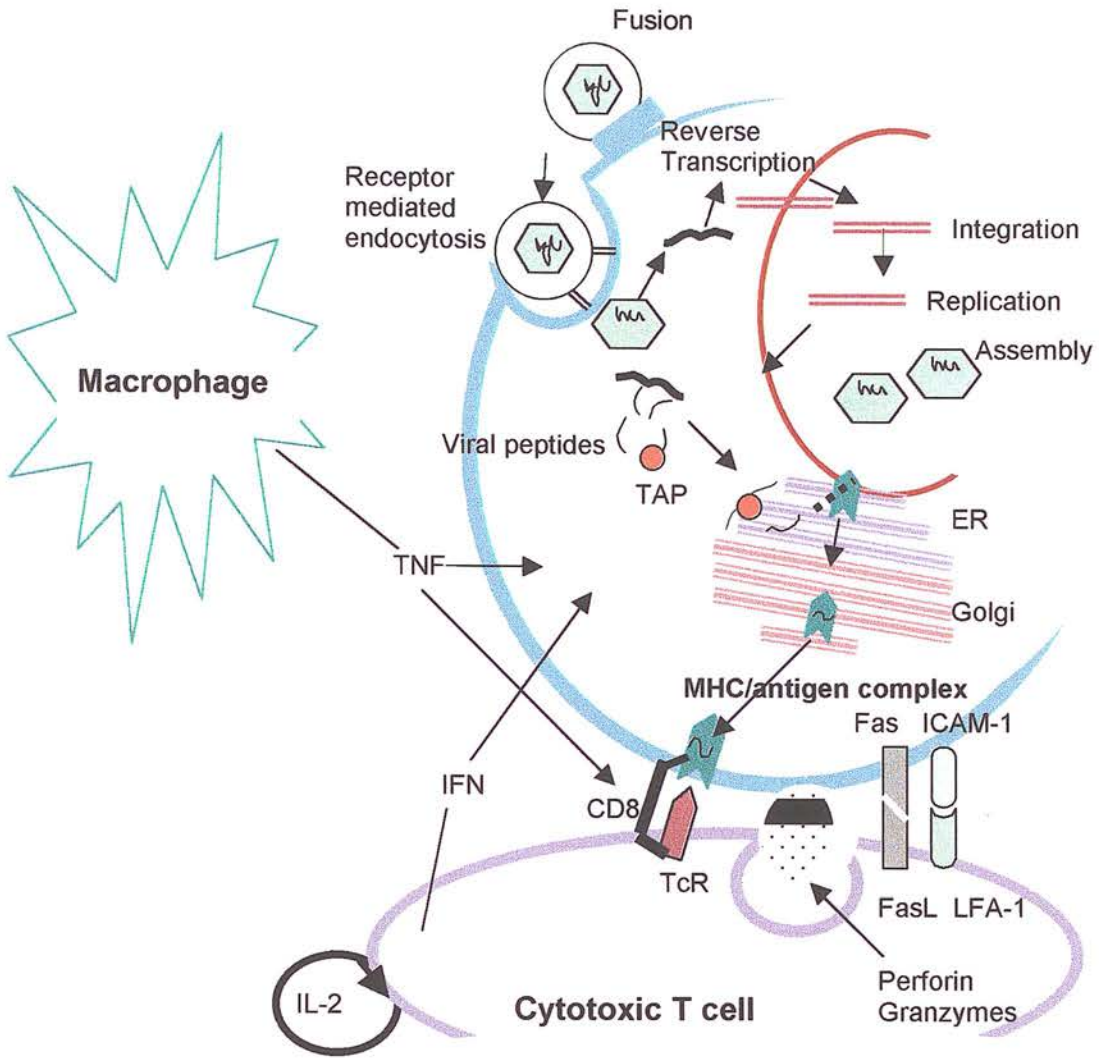


Figure 1.2 Schematic diagram of antigen presentation by major histocompatibility complex (MHC) class I. MHC class I recognises endogenous antigens. On entering the cell viral proteins are cleaved, transported by the TAP proteins and complexed to MHC in the endoplasmic reticulum (ER). MHC/antigen travels to the cell surface for recognition by cytotoxic T cells and/or macrophages. TAP, transporter associated with antigen presentation proteins ICAM, intracellular adhesion molecule; TcR, T cell receptor; IL, interleukin; TNF, tumour necrosis factor; IFN interferon; GM-CSF, granulocyte macrophage – colony stimulation factor.

MHC class II

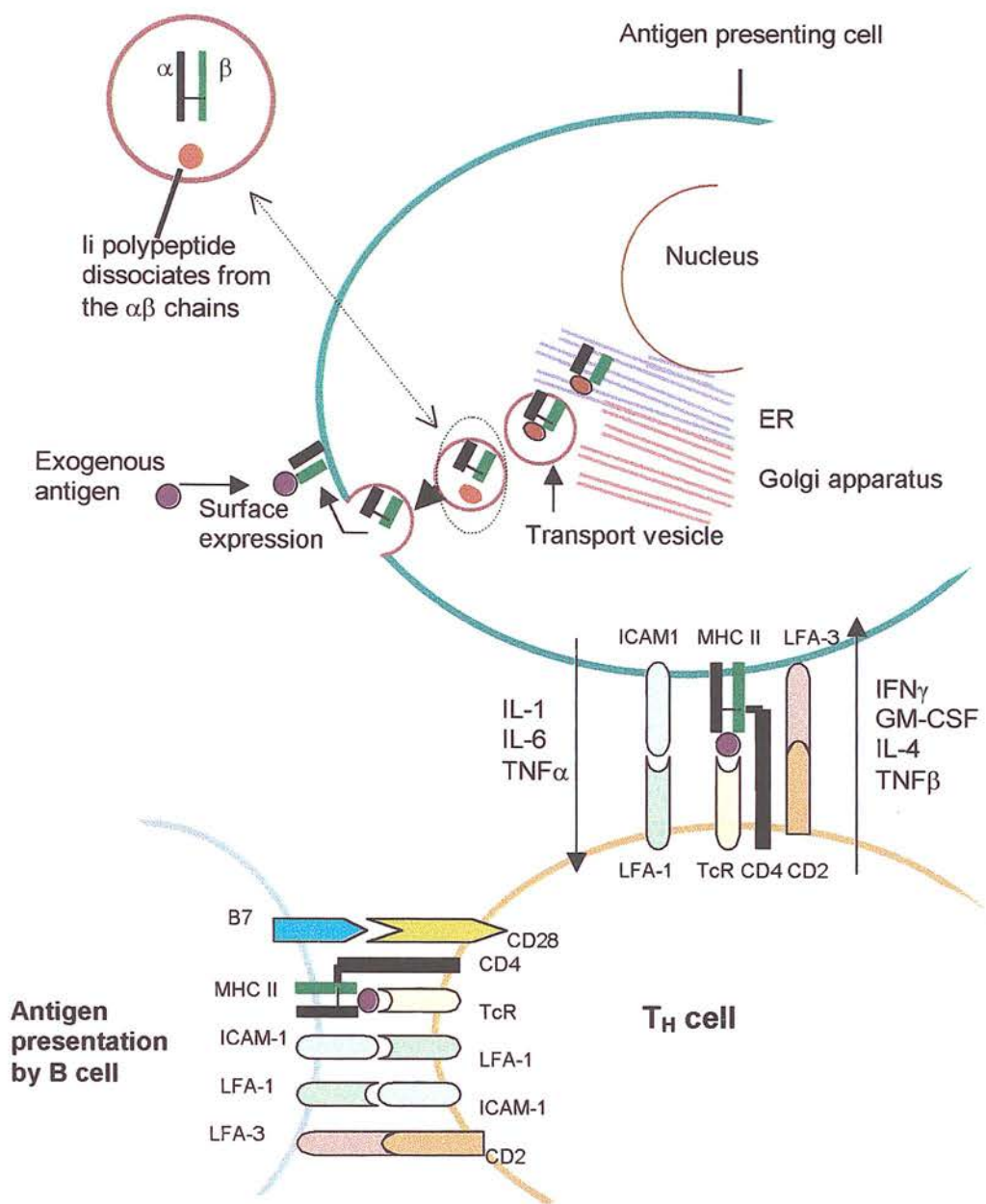


Figure 1.3 Schematic diagram of antigen presentation by major histocompatibility complex (MHC class II). Antigen presenting cells such as macrophages, dendritic cells and B lymphocytes express MHC class II, which recognises exogenous antigens. The MHC $\alpha\beta$ -li complex is formed in and transported through the endoplasmic reticulum (ER) to enter a transport vesicle. The li moiety dissociates and the $\alpha\beta$ chains are transported to the surface of the cell. A stable MHC class II molecule is formed when antigen binds. MHC class II/antigen is expressed on the cell surface for recognition by T_H cells. ICAM intracellular adhesion molecule; LFA lymphocyte functional antigen; TcR T cell receptor; IL interleukin; TNF tumour necrosis factor; IFN interferon; GM-CSF granulocyte macrophage colony stimulation factor.

moiety dissociates from the complex. As the constraints of the MHC class II binding groove are similar to that of MHC class I (detailed above) exogenous antigen entering the cell by endocytosis can be cleaved by the proteolytic enzymes into smaller peptides during transportation to the acidic compartment where they encounter the MHC class II $\alpha\beta$ complex. The dissociation of li facilitates antigen binding, generating a stable MHC class II/antigen molecule that is transported to the surface of the cell where it is recognized by T_H cells.

1.4.2.4 Determination of effector mechanisms

In response to stimulation, naïve T_H cells release a wide array of cytokines. The cytokines expressed by the T_H cell determines whether the effector cells generated will differentiate into T_{H1} or T_{H2} cell types. As the cytokines released by T_{H1} and T_{H2} cells may represent two extremes of the wide pattern of cytokines secreted, categorization into only two types of responses may be an oversimplification. However the T_{H1} response results in T cells that induce macrophage activation and promote cell mediated effector responses. In contrast, differentiation to T_{H2} cells results in helper cells that induce B cell activation and enhance antibody production. The T_{H1} cell type releases IL-2 and IFN- γ , with T_{H2} cells producing IL-4, IL-6 and IL-10. Cytokines such as GM-CSF and TNF- α are released by both T_{H1} and T_{H2} cell types. Once the effector subset has become established the cytokines released can suppress the other cell subset, for example IFN- γ produced by T_{H1} cells inhibits the proliferation of T_{H2} cells, and IL-10 from T_{H2} cells downregulates cytokine secretion by the T_{H1} subset. In a study by Clerici and Shearer (1993) it was reported that a T_{H1} to T_{H2} switch was a crucial stage during HIV infection. They reported that a dominant T_{H1} type response was

detected in reaction to HIV antigens in seronegative, HIV-exposed subjects, whereas a dominant T_H2 type response was evident in the progression to immunodeficiency syndrome (AIDS), characterized by depletion of IL-2 and IFN- γ and increased production of IL-4 and IL-10. It was hypothesized therefore that the T_H1 response conveyed immunoprotection, preventing HIV and/or the progression to AIDS. Furthermore, that any stimuli inducing a T_H2 response could act as cofactor in the development of AIDS.

1.5 Characterisation of JSRV

A serological cross-reaction between the JSRV p25 core protein and p27 core polypeptide of Mason Pfizer monkey virus (MPMV) and mouse mammary tumour virus (MMTV) demonstrated JSRV homology to type B and type D retroviruses (Sharp & Herring 1983). Further evidence characterising JSRV was demonstrated with the advancement of molecular techniques. Initially in 1991, York *et al* successfully isolated and partially cloned the JSRV genome by the synthesis of cDNA from genomic RNA purified from lung washes of infected sheep. Characterization of the clone (JS46.1) substantiated the previously identified homology with the type B and D retroviruses, but in addition, sequences homologous to a human endogenous retrovirus were detected. In contrast, clone JS46.1 did not hybridise to maedi-visna virus, the lentivirus often found in association with JSRV infection.

Viral particles were purified from lung washes of OPA cases and the complete JSRV genome was cloned and sequenced by York *et al* in 1992a and b. It was revealed that the JSRV genome is 7,462 nucleotides long with a genetic organization characteristic of the type B and D oncoviruses: with *gag*, *pro*, *pol* and

env open reading frames (ORF), flanked by the long terminal repeats (LTR) found to be 397 bases, bound by the inverted repeat sequence CTGC at positions 7178 to 7181 and GCAG at positions 122 to 125 (Figure 1.4). The *gag* ORF encodes the JSRV capsid protein (CA), with the Gag precursor identified as a 612-amino acid polypeptide with a molecular weight of 68KDa. The 9.6KDa nucleocapsid protein (NC) of JSRV has two Cys-X₂-Cys-X₄-His-X₄-Cys sequences, separated by thirteen residues, which are thought to mediate binding of the NC to the genomic RNA.

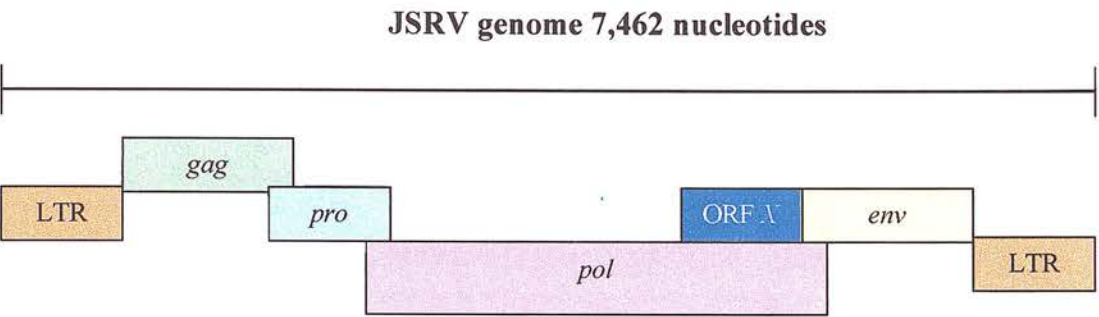


Figure 1.4. Schematic diagram of the complete JSRV genome. Sequencing the JSRV genome identified homology with type B and D retroviruses, characterised by *gag*, *pro*, *pol* and *env* regions flanked by long terminal repeats (LTR). Attached to the *env* region and overlapping *pol* an extra reading frame (*ORF X*) has been identified.

The 326 residue *pro* ORF has a molecular weight of 35KDa. Pol is comprised of two moieties, a protease-like (PrL) domain and the active protease (PR). In the PR cellular aspartyl protease with a core amino acid sequence of Leu-Asp-Thr-Gly has been identified. The PrL and PR are thought to be expressed as a *gag-pro* fusion polypeptide. Integrase, and reverse transcriptase (RT) with the active site composed of the sequence Tyr-Met-Asp- Asp are encoded by the *pol* ORF and are thought to be translated by the expression of a fused *gag-pol-pro* polypeptide. The *env* ORF, which overlaps the *pol* region, encodes the JSRV viral envelope protein precursor. The unglycosylated Env precursor has a molecular weight of 69KDa. The envelope

protein is comprised of two moieties, the surface protein (SU) with an estimated molecular weight of 47KDa and the transmembrane protein (TM), molecular weight 33KDa. Three hydrophobic sections have been identified in the Env precursor, one (13 amino acids) corresponds to the signal peptide, the second (25 amino acids) was located downstream of the SU-TM cleavage site, and the third (24 amino acids) has been identified as the TM anchor, which intersects the membrane and is followed by the cytoplasmic tail. Also within the *env* region of JSRV and overlapping *pol*, an extra open reading frame (*ORF X*) was revealed (York *et al* 1991 & 1992). *ORF X* was found to be unusual in its location within the viral genome and very hydrophobic. It has been predicted that a polypeptide of 166 amino acids could be expressed (York *et al* 1992b), but to date, any product(s) encoded by *ORF X* remain unidentified although it has been established that the endogenous *ORF X* sequence is highly conserved (Bai *et al* 1996). In addition *ORF X* sequences from JSRV isolates from Britain, Italy, Spain and South Africa (Rosati *et al* 2000) were found to highly conserved also. This observation may suggest that *ORF X* could be an accessory gene, advantageous to the virus by bestowing some evolutionary advantage.

1.5.1 Endogenous JSRV proviral sequences

In mammalian systems studied, proviral DNA has been identified integrated into the host genome (Coffin 1982). These DNA sequences, possibly retained by ancestral hosts following viral infections and acquired by Mendelian inheritance through subsequent generations, become part of the genetic makeup of the species. The genome of sheep contains 15 to 20 copies endogenous proviral sequences that hybridise to JSRV probes (Hecht *et al* 1996, Bai *et al* 1996). It was essential therefore to develop a method of distinguishing the endogenous from the exogenous

virus. The compilation of restriction maps identified restriction sites unique to exogenous JSRV proviral DNA, and consequently resulted in exogenous specific molecular markers (Bai *et al* 1996). The *Sca*1 restriction site, located in the *gag* gene of the exogenous JSRV was the first recognised differentiation marker for JSRV (Palmarini *et al* 1996). Furthermore, it was revealed that, although the LTR region of the exogenous and endogenous DNA has approximately 94-98% homology, significant diversity was found in the U3 region. The endogenous JSRV U3 has an average of 78% sequence identity to the exogenous form, but at the 3' 173 bp region of exogenous proviral DNA only 56% sequence homology to the endogenous form was identified. This is as a result of deletions of a total of 47 bases and point mutations in the exogenous form, consequently resulting in a unique U3 sequence (Palmarini *et al* 1996a). In comparison with the highly conserved endogenous *ORF X* sequences collected from Australia, Italy, Britain and South Africa phylogenetic analysis of the *ORF X* sequences derived from lung secretions of OPA cases from Britain, Europe and South Africa revealed three geographically diverse groups. The viral isolate from the South African JSRV appeared to have greater homology to the endogenous sequences than that identified with the European isolates (Rosati *et al* 2000). Palmarini *et al* (2000a) molecularly cloned three endogenous retroviruses of sheep and analysis of their proviral structure, phylogeny, and pattern of expression revealed that all three contained open reading frames for at least one of the structural genes. However, one clone was unable to produce viral particles and the construction of viral chimeras with exogenous JSRV₂₁ detected a defect in the *gag* region of the endogenous sequence. Furthermore major differences from the exogenous JSRV were identified in two short regions in the *gag* (VR1 and VR2) of the endogenous

sequences. A third divergent region (VR3) was identified in the carboxy-terminal portion of the transmembrane protein. In every other respect, the endogenous sequences were found to be very similar to the exogenous (except for deletions and the high level of polymorphism in the U3 region of the LTR described previously). It was shown by Palmarini *et al* (2000a) that the exogenous form of JSRV has the strongest specificity for differentiated epithelial cells of the lung. Transient transfections and reporter assays determined that the LTR of the three endogenous sequences do not have the same tissue tropism as the exogenous JSRV. In contrast it was revealed that endogenous JSRV tropism is strongest in the uterus, with weaker expression in the gut and bronchiolar epithelium of the lung (Palmarini *et al* 2000b, 2001)

It was thought that perhaps the reactivation of the endogenous proviral DNA could result in the expression of exogenous JSRV in neoplastic cells. In that case exogenous JSRV would not be the aetiological agent of OPA but would be expressed as a result of neoplasia. However, JSRV capsid antigen has been detected in tumours, lung secretions, pulmonary lymph nodes and peripheral blood of OPA-affected animals and not in any other tissue of either affected or unaffected animals (Palmarini *et al* 1995, 1996a and 1997). This would suggest that although all sheep have endogenous JSRV, it is the exogenous form of the virus that is directly associated with OPA (Palmarini *et al* 1996a & 1997).

1.5.2 JSRV, the confirmed aetiological agent of OPA

Although substantial evidence implicating JSRV in the development of OPA had been demonstrated, (Martin *et al* 1976, Sharp and Herring 1983, Rosadio *et al* 1988, Hecht *et al* 1996, Palmarini *et al* 1996), the question of whether the exogenous

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form of the virus alone was capable of inducing OPA remained unanswered until recently. In a study by Palmarini *et al* (1999) genomic DNA was isolated from a confirmed OPA case and exogenous JSRV proviral DNA was distinguished from endogenous sequences by JSRV-U3-specific hemi-nested PCR. A recombinant phage was produced with full length exogenous JSRV inserted and this was sub-cloned to create pJSRV₂₁. The plasmid pCMV2JS₂₁ was developed by replacing the U3 region of the upstream LTR with human cytomegalovirus (CMV) promoter. The plasmid was used to transiently transfect 293T cells and the culture supernatant was harvested, shown to contain JSRV and prepared as an inoculum to experimentally infect four neonatal lambs. Two lambs developed histologically confirmed OPA at four months post-inoculation. Evidence that the disease was induced by JSRV₂₁ was demonstrated by exogenous-JSRV-specific PCR, and the presence of the restriction site *Sca*I in the *gag* region, unique to the exogenous form of the virus. Therefore providing confirmation that exogenous JSRV is the aetiological agent of OPA. The development of the infectious molecular clone JSRV₂₁ provided an *in vitro* system, which does not rely on the preparation of virus or infectious particles from natural sources. Using molecular techniques, it is now possible to examine the mechanisms of JSRV oncogenesis and study the pathology of OPA by manipulation of the JSRV genome.

1.5.3 The homology of JSRV with other retroviruses

As stated previously, the homology of JSRV to type B/D retroviruses was established when Sharp and Herring (1983) detected serological cross-reactivity between the JSRV p25 core protein and p27 core polypeptide of the B and D prototypes, mouse mammary tumour virus (MMTV) and Mason Pfizer monkey virus

(MPMV). It has been established that JSRV is morphologically similar to MMTV by the presence of the knoblike glycoprotein spikes on the surface of the envelope (Payne *et al* 1983). Phylogenetic analysis of the JSRV genome has also provided further evidence for homology with MMTV, MPMV, simian retrovirus type I (SRV) and human endogenous retrovirus (HERV-K). JSRV protein sequences were aligned to those identified from the type B/D retroviruses and the level of homology established by the degree of amino acid identity. For example alignment of the JSRV Gag polyprotein with those of MMTV and MPMV identified conserved sequences between the three viruses that correspond to the CA boundaries. Comparison of the terminal COOH of the NC is less clearly aligned but the location of the cleavage site Tyr/Gly-Ala identified in MPMV is similarly positioned in the JSRV Gag (York *et al* 1992b). Overall for JSRV, MPMV and SMRV-type I more than 50% amino acid identity was detected in the Gag, Pol and Pro proteins, but no consistent alignment was detected for the Env proteins. In contrast the JSRV Env proteins were homologous with those from MMTV and HERV-K, but less than 38% amino acid identity was detected between their Gag proteins. A relationship in the structural organization of JSRV and type B Env proteins was evident, with many shared cysteines and glycosylation sites. Only weak similarities between the Env proteins of MPMV, SMRV and JSRV and the type B retroviruses were identified. MPMV and SMRV Env proteins were highly homologous to the type C reticuloendotheliosis virus (REV-A) (Sonigo *et al* 1986).

Similar to JSRV, MMTV infection is also associated with an epithelial neoplasm, but despite the identified homology between the retroviruses, differences in the mode of transmission and localisation of viral transformation are evident.

MMTV is transmitted to the mouse pups *via* ingestion of the virus present in the milk from lactating mothers. The pups are infected during the first two-weeks of life, prior to acidification in the stomach (Acha-Orbea *et al* 1999). Therefore the virus has to be transported from the site of infection to the mammary epithelium, the site of transformation (Figure 1.5).

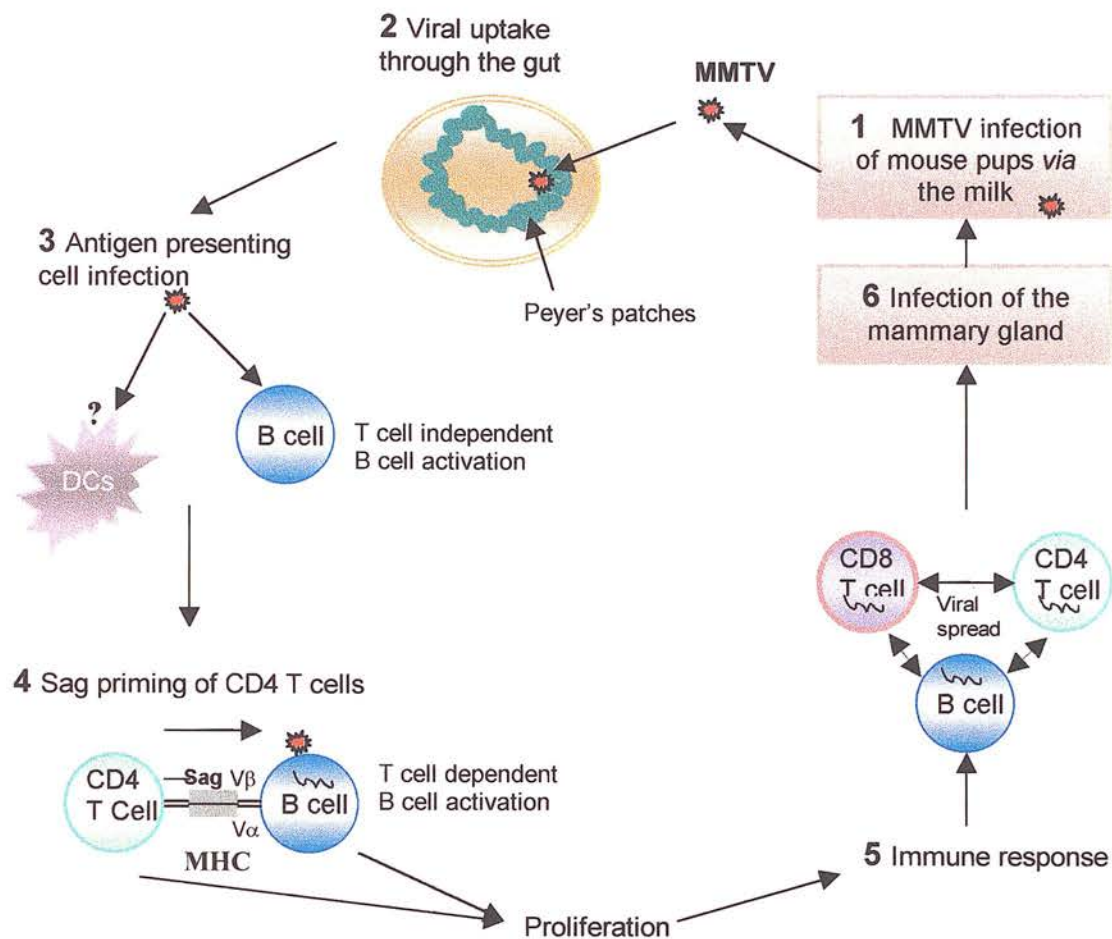


Figure 1.5 Schematic diagram of the life cycle of mouse mammary tumour virus (MMTV). Mouse pups are infected by milk containing MMTV particles. Virus can be detected in the Peyer’s patches in the gut. B cells (and possibly dendritic cells (DC)) are infected an induced to express MMTV Sag, which activates T cells; creating a positive feedback loop that induces proliferation. A chronic immune response is mounted resulting in viral spread and transportation of MMTV to the mammary gland.

This is achieved by viral uptake through the gut epithelium and the subsequent T cell independent infection of resting B cells in the Peyer’s patches (Karpetician *et al* 1994).

The B cells are induced to replicate and express the MMTV superantigen (Sag) (Held *et al* 1993a). MMTV infected B cells then stimulate T cells, which express the Sag-specific V β region. A positive feedback is elicited by T cells cytokine production, which induces the infected B cells to proliferate and consequently increase the MMTV viral load (Held 1993b). The virus is then transported to the mammary gland. Several studies have shown that an intact immune system is required for successful MMTV infection. Luther *et al* (1997) provided evidence to suggest the involvement of antigen presentation in MMTV infection by identifying dendritic cells in close contact with dividing Sag-reactive T cells. However despite the involvement of dendritic cells, mice deficient in B cells could not be infected, and although the lack of Sag-specific T-cells does not prevent MMTV infection, viral amplification cannot occur (Held *et al* 1993a). There is however no evidence to suggest that JSRV carries a super-antigen gene analogous to the MMTV-Sag. In contrast to the transportation requirement of MMTV to the site of transformation, the respiratory mode of JSRV transmission may facilitate direct access to the alveolar epithelial cells, although an initial infection of the lymphoid tissues in the upper respiratory tract cannot be overruled. A disseminated lymphoid infection has been established for JSRV (Holland *et al* 1999). Furthermore, Holland *et al* has provided evidence that dissemination occurs prior to tumorigenesis (this study is described in detail in section 1.6.2).

Tumour of the ethmoid turbinates in the nasal cavity of sheep and goats, enzootic nasal adenocarcinoma (ENA) is caused by the retrovirus nasal adenocarcinoma virus (NAV). Similar to OPA, NAV originates from transformation of secretory epithelial cells and can be experimentally induced by inoculation of cell-

free tumour homogenates (De Las Heras *et al* 1991). Furthermore cross-reactivity with the major capsid protein of JSRV and MPMV suggests that NAV also has type D capsid protein (De Las Heras *et al* 1993). In a study by Cousens *et al* (1996) the *gag* region of NAV from nasal exudates, collected from three different farms, was partially sequenced and found to have 83-92% homology with JSRV-related sequences. Furthermore it was revealed by Palmarini *et al* (2000a) that the *gag* VR1 regions of JSRV and NAV contain a proline-rich region that is absent from the endogenous proviruses. In contrast, polymorphism between JSRV and NAV was detected in the transmembrane V3 region. Also NAV does not possess the *ScaI* restriction site identified in the *gag* region of JSRV. However other restriction sites, specific to NAV have been identified. Therefore despite the high degree of homology between NAV and JSRV, diversity in the two viruses suggests that they are very distinct. Studies are currently underway to sequence the entire NAV genome and characterise the differences, not only between NAV and JSRV but also NAV isolated from goats, caprine nasal adenocarcinoma virus (CNAV) and from sheep, ovine nasal adenocarcinoma (ONAV). Recently it has been demonstrated that the ONAV does not possess the *ORF X* identified in the CNAV and in JSRV (Dr. C. Cousens, personal communication). Furthermore no evidence of a disseminated infection has been identified in the ovine variant (lacking *ORF X*) but dissemination to the lymphoid cells has been detected in the caprine variant (with *ORF X*) (Dr. A. Ortin, personal communication). These preliminary observations may suggest a link between JSRV dissemination and the presence and expression of the *ORF X* gene.

1.5.4 The alveolar epithelial cells targeted by JSRV

The monolayer lining the alveolar surface is composed of two distinct types of epithelial cells, the squamous Type I and the cuboidal Type II pneumocytes. A major role of these cells is to protect the lung from infection. The Type II pneumocytes and non-ciliated (Clara) cells are the only cells identified to date that are targeted by JSRV to undergo viral transformation ((Jassim 1988, Palmarini *et al* 1995, DeMartini and York 1997). The type II pneumocytes, in addition to being an integral component of the alveolar epithelial layer, have several unique biological functions. Firstly they are responsible for the synthesis, storage and secretion of alveolar surfactant. The secretory function of the pneumocytes may be augmented by viral transformation, detected by the accumulation, and often copious quantity of fluid found in the lungs and respiratory tract of many affected animals. Secondly they maintain the integrity of the alveolar interstitium by regenerating and replacing Type I pneumocytes that have been damaged during lung injury. Thirdly studies have suggested that the alveolar epithelium may have immunomodulatory functions and play a role in determining immune responses in the lung. Studies of murine and rat models with acute interstitial lung disease have detected immunoreactivity with the expression of pro-inflammatory cytokines such as tumour necrosis factor- α (TNF- α), transforming growth factor- β (TGF- β), interferon- γ (IFN- γ) and interleukin-4 (IL-4) expressed by the type II pneumocytes (Kapanci *et al* 1995, Wallace and Howie *et al* 1999). In rats the type II pneumocytes have been found to produce monocyte chemoattractant protein-1 (MCP-1) in response to IL-1 stimulation (Paine *et al* 1993), and can be induced to produce a neutrophil chemoattractant, thought analogous to IL-8 (Crippen *et al* 1995). In a study of human alveolar epithelial cells

by Eghtesad *et al* (2001), the constitutive production of MCP-1 by type II pneumocytes was demonstrated and amplification of MCP-1 expression by stimulation with TNF- α was also established. Eghtesad *et al* (2001) also reported that the alveolar epithelial cells could induce the transendothelial migration of T cells and monocytes by participation in the local cytokine network within the lung. Furthermore the alveolar epithelial cells contribute to immunomodulation of inflammatory response by the expression of chemokines and biologically active mediators, which modulate the recruitment of alveolar macrophages and leukocytes into the microenvironment. It could be hypothesised therefore that the biological and immunological functions of the cells targeted for viral transformation, may themselves play an active role in determining the immune response during OPA tumorigenesis.

1.5.5 The cell-surface receptor for JSRV

Retroviral entry into a cell is initiated by the interaction of the viral envelope and a specific receptor present on the surface of the cell. The identification of the cell-surface receptor for JSRV was determined by using chimeric pseudotyped virus containing the *gag* and *pol* proteins from Moloney murine leukaemia virus, the *env* protein of JSRV and encoding human alkaline phosphatase (AP) as a detection marker for viral transduction (Rai *et al* 2001). Sheep and human cells (except HeLa cells) were transduced by this construct, but as rodent cells were not, this suggested that they do not possess a functional receptor for JSRV *env*. This facilitated phenotypic screening of human/hamster hybrid cell lines, which identified several closely linked markers localised to the p21.3 band of human chromosome 3. The JSRV receptor mapped to a region, which had previously been sequenced in a search

for cancer suppressor genes. This was identified as HYAL-2, a glycosylphosphatidylinositol (GPI)-anchored cell-surface protein (Rai *et al* 2001). GPI-proteins are found on the apical surface of epithelial cells and although HYAL-2 is related to known hyaluronidases the function of HYAL-2 is unknown. For cells expressing HYAL-1, hyaluronidase activity has been demonstrated, but in contrast no hyaluronidase activity was evident in cells associated with HYAL-2. It was demonstrated that if HYAL-2 was removed the cells became resistant to JSRV infection. Furthermore, it was found that over expression of the rodent HYAL-2 resulted in infection of rat cells and NIH 3T3 mouse cells, which do not normally have a functional receptor for JSRV. The generation of transgenic rodents with functional HYAL-2 receptors would advance the study of JSRV infection and oncogenesis by providing a laboratory rodent model.

1.6 Transmission of JSRV

1.6.1 Early transmission experiments

The first report that OPA was an infectious disease was described by De Kock in 1929, when he performed transmission experiments by cohabitation of affected and unaffected sheep. However, his findings were not conclusive as OPA lesions were detected in only one animal. Notwithstanding, the hypothesis that OPA was a contagious disease persisted and transmission experiments continued. The cohabitation experiment was successfully repeated, with OPA lesions detected in six out of eight incontact sheep (Dungal *et al* 1938). These findings provided the first compelling evidence for the transmission of OPA. To further substantiate the transmission theory a series of experiments were conducted, which included healthy sheep being inoculated with blood and fed faeces from affected animals. These experiments were unsuccessful. In contrast, when healthy and affected sheep were

housed in head to head contact, OPA was transmitted, therefore exhaled air was thought to be the source of infection. The exhaled air was collected into glycerine and administered to healthy sheep as an intratracheal or intrapulmonary inoculum. OPA lesions were identified at necropsy four to eight months post-inoculation (Dungal 1946). Thus during natural infection the mode of transmission is suspected to be inhalation of aerosols from infected flockmates.

1.6.2 Experimental JSRV infection

Transmission of OPA to healthy lambs has also been achieved by using material derived from OPA tumours. Lesions were identified in seven out of twenty-two sheep experimentally infected with cell-free suspensions by incontact, aerosol and inoculation (Markson and Terlecki 1964). In a similar study by Enchev (1966) using cell-free suspensions, five of sixteen sheep developed OPA lesions.

Initially during experimental infection a long lag phase before the development of clinical disease was required. For example in the studies described above (Markson and Terlecki 1964, Enchev 1966) clinical symptoms were not apparent until 19 and 23 months post-inoculation, respectively. In many studies clinical signs of OPA could only be detected after an incubation period of two to four years. Wandera in 1970 determined that the route of inoculation and the age of the animals were important factors in determining the incubation period. Experimental infection of lambs resulted in reducing the time to six months. The incubation time was further reduced by using an inoculum from partially purified, concentrated lung fluid. When inoculated intratracheally clinical disease was induced in neonatal lambs within three to four weeks (Sharp *et al* 1983). At the present time, under experimental conditions clinical and pathological OPA can be induced in neonatal

lambs by intratracheal inoculation with virus purified from tumour (Martin *et al* 1976), lung fluid (Sharp *et al* 1983) or JSRV infectious molecular clones (Palmarini *et al* 1999, DeMartini *et al* 2001).

1.6.3 Evidence for vertical transmission

Recently evidence of possible vertical transmission of OPA has been demonstrated, with JSRV detected in foetuses from three histologically confirmed OPA cases (De las Heras *et al* 2000). Five foetuses, with a gestational age of 16 weeks, were removed from one Spanish dam, and four foetuses, with a gestational age 15-19 weeks, from two Scottish dams. No macroscopic or microscopic evidence of OPA was detected in the lungs, mediastinal lymph node or thymus of any of the foetuses, and immunohistochemistry did not detect JSRV capsid antigen (JSRV-CA). However, exogenous JSRV was detected by PCR in the lungs of four of the five Spanish foetuses and in the PBMC from two of the four Scottish foetuses (from the same dam). Further evidence to support JSRV infection *in utero* was demonstrated in the same study by the following. Histological examination of lung tissue collected from a three-day-old lamb revealed well-developed lesions characteristic of OPA tumour. Immunohistochemistry detected JSRV-CA in the lung, and JSRV was identified by PCR of DNA, excised from the lung tissue. The mother of this lamb was traced and although no macroscopic, microscopic or immunohistochemical evidence of OPA or JSRV-CA was found, JSRV was detected in the PBMC by PCR. The extent of tumour development in the three-day-old lamb suggested that it was infected *in utero*. It has been suggested that infection of the foetus arises from the dam *via* the placenta, although the mechanism of viral transportation across the placenta is not known (De las Heras *et al* 2000). Parker *et al* (1998) investigated the

the possibility of OPA-free lambs born from OPA-affected ewes by embryo transmission experiments. They found no evidence of infection in two to six day old embryos, suggesting that infection of the embryo does not occur until a later point in the gestation period.

1.7 Pathogenesis of OPA

The first pathological description of OPA was given in South Africa, by Mitchell in 1915. Today, OPA is classified as a contagious low-grade adenocarcinoma. Enlargement of the lungs, the development of neoplastic lesions and, in many cases, the production of lung fluid are the pathognomonic features of OPA (Sharp and Angus 1990). Two pathological forms of OPA have been identified, classical and atypical, and there appears to be a geographical distinction in the prevalence of the tumour type.

1.7.1 Clinical symptoms

The clinical signs of classical OPA are shallow breathing, an increase in respiration, exacerbated by any physical exertion, which causes the animal to pant and heave at the flanks. If OPA is advanced, moist rales can be detected, arising from the production and accumulation of fluid in the lungs, which can often be detected as a nasal exudate. Lung fluid is a liquid comprised of mucus, lipids, surfactant proteins and cells characterised mainly as macrophages (Palmarini 1995). It has also been reported that lung fluid is a major source of IgA (Smith *et al* 1975). The lung fluid can be collected from OPA cases by raising the hindquarters of the animal above the head allowing the fluid to pour from the nostrils (Figure 1.6). Lung fluid, collected from OPA cases (Figure 1.7) is a major source of JSRV, which can be partially purified as detailed in Chapter 2, section 2.1.3.



Figure 1.6. Wheelbarrow technique for the collection of lung fluid from OPA affected sheep. By raising the hindquarters of the animal the lung fluid draining from the nostrils can be collected.



Figure 1.7. Collection of lung fluid from a sheep with OPA.

The volume of lung fluid produced varies from animal to animal but in some cases up to 500ml per day has been collected. The production of lung fluid has long been recognised as a field test in the diagnosis of OPA (Stevens 1957). However, not all OPA affected sheep produce lung fluid, and fibrosed lesions are identified in the lungs of ‘fluid-free’ sheep at necropsy (Sharp 1981).

An increase in temperature is only detected in OPA cases in the terminal stages of disease, when a secondary infection is evident also. Appetite is not reduced in the OPA affected sheep, but the respiratory symptoms are accompanied by weight loss, with severe dyspnoea evident in advanced cases (Figure 1.8). Clinical signs of classical OPA inevitably result in progressive respiratory distress and the death of the animal. In contrast, the atypical form OPA usually remains subclinical, with OPA tumours only detected at necropsy performed during the diagnosis of unrelated illnesses (Gonzalez 1990), or identified during abattoir studies (De las Heras 1992).



Figure 1.8 Adult sheep in the terminal stages of OPA. Severe weight loss is a symptom of OPA.

1.7.2 Classical OPA

The classical form of OPA is commonly described in Scotland (Hunter and Munro 1983, Sharp and Angus 1990), South Africa (Tusin 1969) and Israel (Nobel 1958). Furthermore in 2001, Sanna *et al* described classical OPA in archive tissues collected during the 1980s outbreak of OPA in Sardinian Moufflon (*Ovis musimon*), a wild sheep closely related to the domesticated sheep (*Ovis aries*) (Neiddu *et al* 1987).

1.7.2.1 Gross pathology of classical OPA

In the initial development of classical OPA tumours observed macroscopically, the lesions are small, grey/white nodules of varying size, which are raised slightly from the surface of the lung. The nodules exist either as individual foci or have become confluent. In advanced cases the lungs are always greatly enlarged, in some instances to three or four times the normal size (Figure 1.9), and when examined within the thoracic cavity they may be attached to the cavity walls by connective tissue.

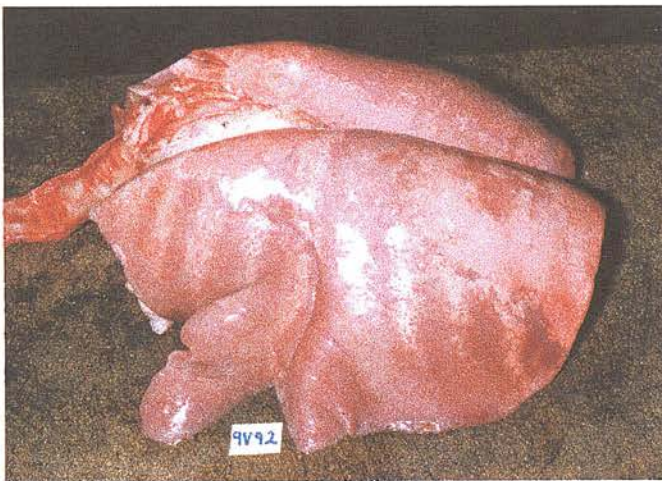


Figure 1.9. OPA Lungs. The lungs from sheep affected by OPA are often greatly enlarged. The striated appearance on the surface of the lungs shown here is due to pressure on the rib cage during respiration.

The tumour mass is firm to touch, grey in colour and can be detected in any or all lobes of the lungs (Figure 1.10a). When the lung is cut the tumour has a pink/grey appearance and a white exudate can often be detected oozing from the cut surface (Figure 1.10b). When present, lung fluid aerated by respiration is observed in the bronchi and bronchioles and pours from the trachea as a white frothy fluid, shown in Figure 1.10c (Dungal *et al*, 1938, Cuba-Caparo *et al* 1961, Wandera 1971, Gonzalez 1990, Sharp and Angus 1990).

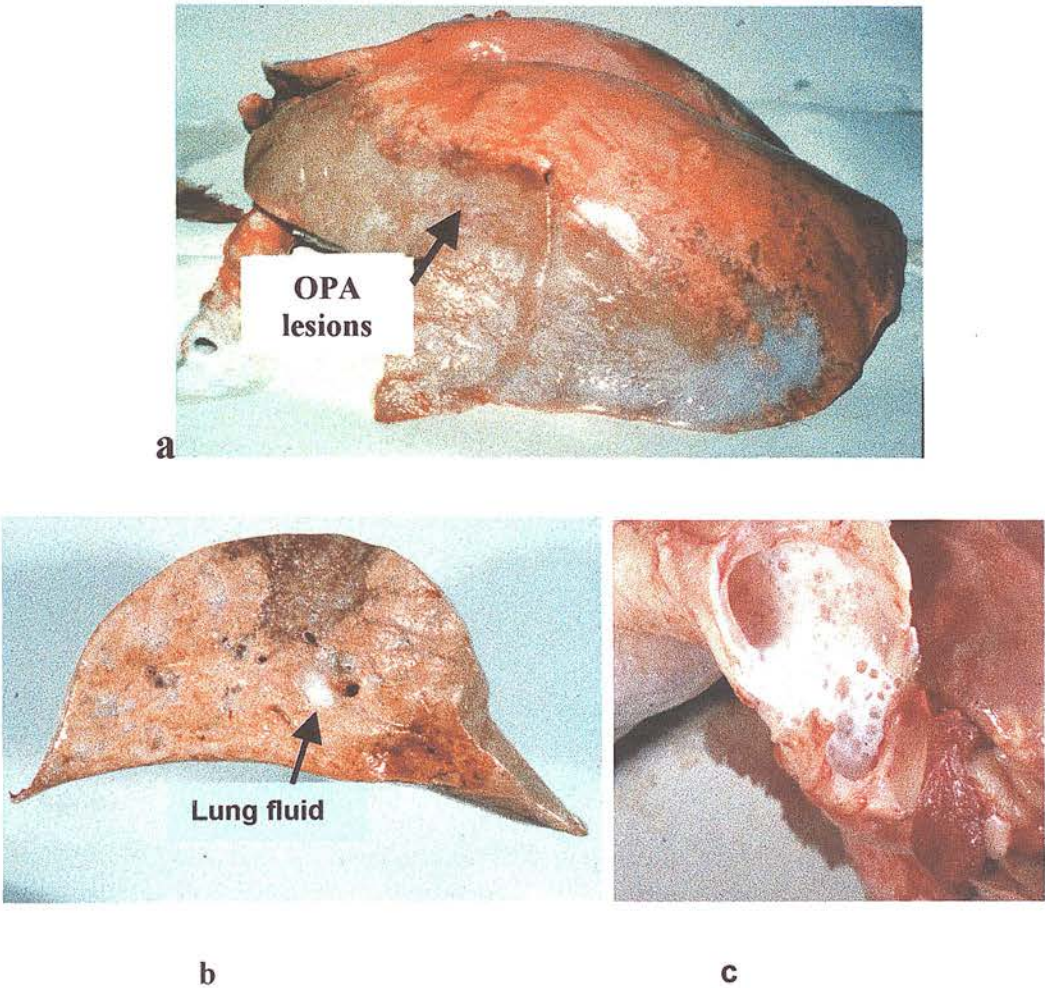


Figure 1.10. Gross pathology of classical OPA lungs. OPA lesions can be detected as small individual nodules or as shown in Figure 1.10a, a large confluent, grey/white tumour mass. Figure 1.10b shows the cut surface of the classical OPA lung. Lung fluid observed in the trachea as a white frothy liquid is shown Figure 1.10c.

1.7.2.2 Histopathology of classical OPA

Histopathological examination of the classical OPA tumour shows a single layer of neoplastic cuboidal or columnar epithelial cells lining the airspaces of the alveoli or bronchioles. The tumour is surrounded and supported by stromal tissue, which becomes thicker and more fibrous as the tumour ages. Tumour nodules increase in size due to the spread of type II pneumocytes along the alveolar walls. Inflammatory infiltrates are often detected in the region of the tumour with numerous neutrophils and enlarged macrophages identified within the tumour and surrounding parenchyma (Nobel *et al* 1971, Perk 1982, Sharp and Angus 1985, 1990 and DeMartini 1997). As alveolar macrophages have been identified in early, uncomplicated lesions it is unlikely that their presence is due to secondary infection. Reports from various studies have also identified lymphocytes and plasma cells in the stroma of the tumour (Dualde-Perez 1966, Hod 1977, De las Heras 1995, Garcia-Goti 2000).

1.7.3 Atypical OPA

In Spain (Aragon and the Basque Country), although classical OPA is recognised, the atypical form of OPA has also been described. The pathology of atypical OPA was described by Dualde-Perez (1966), Gonzalez (1990) and De las Heras *et al* (1992), though in these reports the term ‘atypical OPA’ was not used. Similarly in Peru, the OPA lesions described by Cuba-Caparo (1961) were of the atypical form of OPA. Both forms of OPA have been identified, not only in the same flock of sheep, but also in the same lung (Gonzalez 1990).

1.7.3.1 Gross pathology of atypical OPA

Atypical OPA lesions differ from the classical form macroscopically as the tumours are seen as individual, small, round, hard nodules located in the diaphragmatic lobe. When the lung tissue is cut the nodules are dry and white in colour (Figure 1.11). There is no evidence of fluid in the bronchi, bronchioles or trachea (De las Heras 1992).

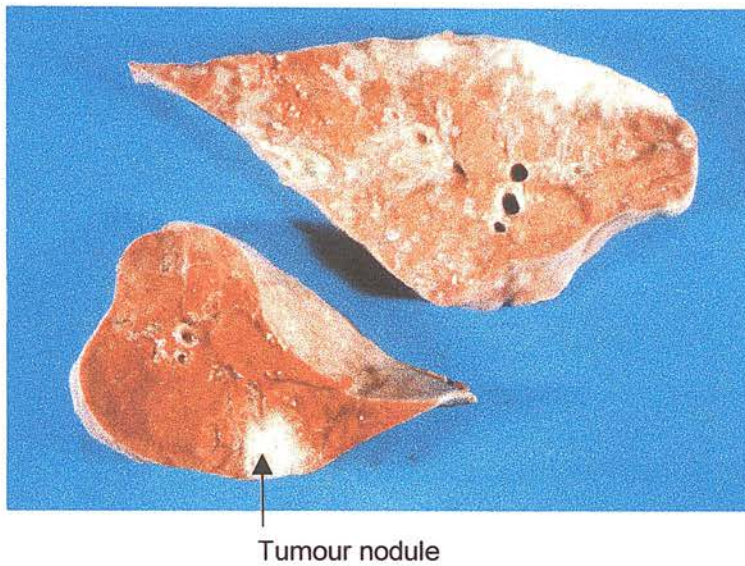


Figure 1.11 Gross pathology of atypical OPA lung. The OPA lesions are observed as dry, white nodules with no evidence of lung fluid on the cut surface of the lung.

1.7.3.2 Histopathology of atypical OPA

Histopathological examination of the atypical OPA tumour reveals histological similarities to those of the classical form, but a more distinctive differentiation between the neoplastic region and the surrounding parenchyma is observed. Microscopic tumours can be identified as distinct foci; often surrounding the larger macroscopically observed nodules. Mononuclear cells have been identified in association with the bronchioles and non-neoplastic regions of the OPA lung.

Furthermore mononuclear cells characterised as lymphocytes and plasma cells have been detected infiltrating the stroma of the tumours to a much higher extent than in the classical form of OPA (Dualde-Perez 1966, Hod 1977, De las Heras 1995, Garcia-Goti 2000). In contrast the large infiltration of neutrophils detected in classical OPA is rarely found in association with atypical OPA.

1.7.4 Two distinct forms of OPA or diverse pathology of the same disease

As stated earlier, both forms of OPA have been identified in the same lung, and the exogenous-specific JSRV U3 LTR and the JSRV-capsid antigen have been detected in the lungs of sheep with classical or atypical forms of OPA. Furthermore atypical and classical OPA could not be distinguished by sequencing the JSRV LTR region (Garcia-Goti *et al* 2000). The absence of lung fluid, distinctive morphology and the quantitatively larger population of immune cells present may account for the asymptomatic nature of the atypical form of OPA. The greater infiltration of immune cells into the atypical tumour stroma may be indicative of an immune response, arresting tumour development or, as suggested in a report by De las Heras (1990), that the two forms of OPA may not be different diseases but the diverse pathology of the same disease. Also, Garcia-Goti *et al* (2000) when characterising the two pathological forms of OPA, suggested that the differences distinguishing the two forms ‘may represent the two extremes of a disease spectrum, rather than two separate forms of the disease.’

1.7.5 OPA Metastasis

OPA metastasis has also been previously reported, with spread to the regional draining lymph nodes being the main organs affected. In contrast metastasis to the extrathoracic regions is more infrequent (Rosadio *et al* 1988a, Bouljihad *et al* 1996

Palmarini *et al* 1995,1996b & 1997). There is general disagreement regarding the frequency of metastasis in OPA. The relative infrequency of this occurrence in sheep examined in many parts of the world may simply reflect the reduced lifespan of OPA affected animals. In contrast, a high rate of metastasis has been reported in Awasi (fat-tailed) sheep but this may represent a genetic predisposition for metastatic spread in this breed (Rosadio *et al* 1988).

1.8 JSRV and ovine immunity

1.8.1 The lack of humoral response

The immunological response to JSRV infection is at present poorly understood. During JSRV infection and development of OPA a controversial issue immunologically has been the absence of a humoral response. In the 1983 study by Sharp and Herring, no JSRV-p25-specific antibodies were detected in the sera from seven adult OPA cases in the terminal stages of diseases, nor from two experimentally infected lambs. Subsequent studies have also failed to detect a JSRV-specific antibody response (De Martini *et al* 1988, Verwoerd 1990). In contrast to these experiments using natural material for the detection of an antibody response, seropositivity was reported to a recombinant Mason-Pfizer monkey virus (MPMV) p27 capsid antigen-glutathione S-transferase (GST) fusion protein (GST-p27). The fusion protein was constructed and used as an antigen in the development of an enzyme-linked immunosorbent assay (ELISA) (Kwang *et al* 1995). The cross-reactivity of the GST-p27 fusion protein to OPA was tested by Western blot, with positive reactions detected in the sera of seven (100%) OPA positive sheep, and no reaction was detected in twenty-three OPA-negative controls. ELISA serodiagnosis of OPA detected 15.6% seropositivity in 160 visibly healthy sheep, 30% in 40 sheep

with chronic respiratory disease symptoms, (all from USA), and 2.7% of 73 healthy sheep and 45% of 96 previously confirmed OPA cases in Italy. An evaluation of the detection of JSRV-specific antibodies was re-examined in 1998 by Ortin *et al* to ascertain if antibody responses to recombinant JSRV-CA could be used as a diagnostic tool to identify OPA or enzootic nasal tumour (ENT) cases, by comparison with animals free of those infections. A recombinant JSRV-CA, with 100% amino acid homology with ENTV, was expressed as a GST fusion protein and detected seropositivity in OPA and ENT cases (confirming the findings of Kwang *et al*), but a positive reaction was also detected in sera from histologically confirmed healthy animals, from flocks with no history of ENT or OPA. Further reservations as to the specificity of the reaction appeared when seropositivity was detected in the sera from seven out of eight sheep from the Falkland Islands and Australia; countries where OPA and ENT have not been reported. Evidence confirming that this reaction was not OPA or ENT specific was demonstrated by absorption with a lysate of *E. coli* containing GST but no JSRV (or ENTV related) proteins. This resulted in the elimination of positive reactions in all sera tested, whereas absorption with JSRV-CA resulted in a loss of reactivity in only a few animals. Therefore the likely explanation for the positive reactions was that seropositivity was a response to prokaryotic proteins or to the GST moiety of the construct. This study therefore supported the earlier findings that no JSRV-specific circulating humoral response was detected.

1.8.2 JSRV disseminated lymphoid infection

As described in section 1.3.6 the cells targeted for viral transformation are the type II pneumocytes and non-ciliated Clara cells in the lungs, but these are not the only cells infected by JSRV. Palmarini *et al* (1996b) identified a JSRV disseminated

infection of the lymphoid tissues of sheep affected by OPA. In 1999, Holland *et al* demonstrated the presence of proviral DNA or RNA in lymphoid cells, purified from the mediastinal lymph nodes of eight SPA cases. This was further characterised by identifying the lymphoid cells infected by JSRV, estimating the proviral load detected in each subset and establishing if the lymphoid infection could be detected prior to tumorigenesis (Holland *et al* 1999). Macrophages were recovered by adherence and the lymphocyte subsets were isolated by miniMACs selection using a panel of monoclonal antibodies specific for CD4, CD8 and B cells (sIg). The frequency of JSRV proviral DNA was determined by limiting dilution analysis (LDA) using the exogenous JSRV U3-specific hemi-nested PCR. It was revealed that JSRV proviral DNA was distributed in all the subsets examined. The estimated mean proviral load was found to be greatest in macrophages with one copy of JSRV provirus per 2,526 cells, followed by B cells (1copy/3,768 cells), CD4 T cells (1copy/6,825 cells) and CD8 T cells (1copy/16,683 cells). Analysis of the peripheral blood mononuclear cells (PBMC) from eight animals estimated a JSRV proviral load of less than 1 copy/240,000 cells. As only a maximum of two of the eight replicates tested positive this could indicate that the proviral load in the blood is too low for consistent detection or may suggest that JSRV infection in the blood is transient. Immunohistochemistry, using the same monoclonal antibodies, was performed to identify any alteration in the phenotypic profile of the mediastinal lymph node and the blood as a result of JSRV infection. In the peripheral blood mononuclear cells (PBMC) from the eight OPA cases a CD4 T cell lymphocytopaenia was detected, in accordance with previous findings (Rosadio and Sharp 1992), in addition an overall increase in the total number of leukocytes was counted in the OPA cases, in

comparison with uninfected sheep (number of control animals was not stated). In the same study it was established from the detection of JSRV proviral load in the blood and mediastinal lymph nodes of thirteen experimentally JSRV infected neonatal lambs that the disseminated infection occurred as an early event, prior to the detection of clinical disease. Hemi-nested PCR detected JSRV provirus in the mediastinal lymph node of each of the thirteen lambs between seven and twenty-eight days post inoculation, although only one to five out of eight replicates were positive. OPA was histologically confirmed in one lamb at twenty-one days and one lamb at twenty-eight days post inoculation, confirming that JSRV dissemination to the lymphoid cells occurred as an early event, preceding tumour development. During this experiment the PBMC were tested also for the detection of JSRV provirus with only two of the thirteen samples testing positive (1/8 and 2/ 8 replicates), providing evidence that the proviral load in the peripheral blood is very low during both natural and experimental JSRV infection. This study therefore provided evidence of dissemination to the lymphoid system and the peripheral blood in naturally infected OPA cases, in the terminal stages of disease. Furthermore, the major cell subsets infected were identified and it was established that JSRV infection of the lymphoid cells occurs as an early event during experimental JSRV infection. It remains to be established whether JSRV dissemination precedes or follows transformation of the epithelial cells in the lung.

A comparative study of classical OPA, atypical OPA, in-contact sheep and sheep from non-affected flocks was performed to detect JSRV proviral DNA during the pre-clinical stages of naturally transmitted OPA (Gonzalez *et al* 2001). JSRV proviral DNA was detected by exogenous U3-specific hemi-nested PCR of

peripheral blood and samples of brain, kidney, mammary gland, mammary lymph node, spleen, mediastinal lymph node, non-tumour region of lung and tumour (where present). JSRV proviral DNA was detected in the peripheral blood leukocytes (PBL) of 100% of the classical, 83% of the atypical OPA cases, 40% of the in-contacts, and was not detected in any of the control sheep. The data was further analysed to determine the differences in the number of positive replicates between the four groups of sheep. The control group was not considered, as there was no JSRV proviral DNA detected. No difference was detected between the groups in the mammary gland, brain and kidney (examined collectively as a single group – extra-thoracic non-lymphoid (ENL) organs). The lung tumour samples were all positive in the classical and atypical OPA groups. From the way in which the data is expressed it is not possible to determine the group number of positive PBL replicates, as the PBL samples were included with non-neoplastic lung, spleen, mediastinal and mammary lymph node. The results showed that JSRV was detected in the blood of four out of ten in-contact sheep, in the absence of histologically evident OPA, but positive PCR was detected in only one out of eight replicates for three of the animals. Therefore, as with the Holland *et al* (1999) study, this is suggestive of the low viral load identified in the peripheral blood.

1.8.3 Lymphocytopaenia and neutrophilia

In a study by Rosadio and Sharp (1992) the phenotypic profiles of the peripheral blood leukocytes from six OPA adult ewes were characterised and compared with three uninfected control animals. Overall, a reduction in the absolute number of total lymphocytes was detected in the OPA cases in comparison with the controls. The major leukocyte frequency alteration was identified as a reduction in

circulating T cells, characterized by a CD4⁺ lymphocytopaenia. The absolute number of CD8 T cells and B cells were found to be elevated in the OPA cases but the percentage was reduced in comparison with the controls. This study also measured leukocyte frequency alterations in three lambs aged five – six weeks old, which had been experimentally infected with JSRV, and compared the phenotypic profile with that of two uninfected, age-matched control lambs. Leukocyte frequency alterations were also reported for the lambs. However, from the data presented a reduction of only 1.8% positively labelled CD2 T cells was detected in the infected lambs compared with the controls, although the differential in the absolute numbers of positive CD2 T cells was greater. However, the CD4 T cells were reduced by 3.5% in the JSRV-infected lambs compared with the control lambs. The percentage of CD8 T cells detected in the JSRV-infected lambs was increased, but the number of positively labelled CD8 T cells was not altered between the two groups. An increase was also detected in the percentage and numbers of B cells in the JSRV-infected lambs compared with the controls.

The preliminary findings of Rosadio and Sharp (1992) included an evaluation of cells detected in bronchioalveolar lavage (BAL) from nine adult OPA cases, compared with six uninfected adults, and also three experimentally JSRV-infected lambs were compared with two age-matched controls. A 24-fold increase in the influx of immune cells was identified in the BAL from the adult OPA cases, compared with the non-OPA adult sheep although the percentage of alveolar macrophages was comparable between the two groups. In contrast an increase in the total lymphocyte and polymorphonuclear cell populations were detected in the OPA cases. Characterization of the pulmonary leukocyte subsets revealed a decrease in

CD2 and CD4 T cells and increased CD8 T cells and B cells from the BAL from the OPA cases in comparison with the control sheep. A 34-fold increase in the influx of immune cells was detected in the BAL from the experimentally infected lambs compared with the controls. The percentage of alveolar macrophages was reduced in the JSRV-infected lambs compared with the controls but the number of macrophages was greatly increased. No alteration between the two groups was detected in the number of total lymphocytes, and the number and percentage of polymorphonuclear cells were also increased in the JSRV-infected lambs. Characterization of the pulmonary leukocyte subsets revealed a decrease in CD2, CD4 and CD8 T cells and an increase of only 0.9% in B cells from the BAL from the JSRV-infected lambs in comparison with the BAL from the controls. Unfortunately, the statistical significance of the reported data for frequency alterations detected in the peripheral blood and BAL was not stated. The preliminary findings of a peripheral lymphocytopaenia was demonstrated from data generated at only one time point during the experimental JSRV infection and from only three JSRV-infected and two control lambs. Therefore an extended investigation into leukocyte frequency alterations during JSRV infection is required.

1.9 Project aims

It was the aim of this project to further the understanding of the relationship between JSRV and ovine immunity by identifying any immunological responses or alterations in the systemic immune system following JSRV infection. As a peripheral CD4 T cell lymphocytopaenia and neutrophilia previously have been identified in terminal OPA cases (Roasadio and Sharp 1992) it was hypothesised that any immune responses in JSRV infected animals may be detected systemically. This

hypothesis was tested by monitoring the phenotypic profile of the peripheral blood leukocytes and cell-mediated responses at all stages of disease from JSRV inoculation, through tumorigenesis and into the terminal stages of OPA. The immunological status in the terminal phase of the disease was determined by the phenotypic characteristics and cellular responses in the blood of naturally infected field cases, compared with those of non-OPA adult sheep. Clinical and pathological OPA was experimentally induced in neonatal lambs by intratracheal inoculation with virus purified from lung fluid (Sharp *et al* 1983). Thus monitoring phenotypic and functional changes in the peripheral blood during experimentally induced tumorigenesis was possible. Furthermore to determine whether the demonstrated lymphocytopenia, neutropenia or as yet unidentified frequency alterations were early events during JSRV infection or occurred post-tumour development. To test the hypothesis that local immune responses may occur in the OPA lung, the identification of tumour infiltrating cells and alterations in their distribution patterns were investigated by comparative immunohistochemical analysis of immune cells in the lungs of confirmed OPA cases and control animals.

To test these hypotheses it was proposed that

☛ The phenotypic frequencies in peripheral blood mononuclear cell (PBMC) subsets be monitored using a panel of monoclonal antibodies specific for CD2⁺ T cells, CD4⁺ T cells, CD8⁺ T cells, $\gamma\delta$ T cells, B cells and MHC class II antigens, with the results determined by flow cytometry. Neutrophils would be counted also.

☛ To determine whether any observed leukocyte frequency alterations occur as early events during JSRV infection, during tumour development or are restricted to the terminal phase of the disease.

☒ Alterations in cellular function, during JSRV infection would be determined by monitoring non-specific *in vitro* lymphoproliferation to mitogen stimulation, to identify changes in cell-mediated responses in the peripheral blood of JSRV-infected animals.

☒ ***In situ*** phenotyping of the OPA lung would provide preliminary identification of tumour infiltrating lymphocyte subsets. Furthermore comparative analysis between the non-neoplastic region of the OPA lung and lung tissue derived from uninfected control animals would identify any influx of immune cells into the tumour region and demonstrate any alteration in the distribution pattern of immune cells within the OPA lung.

Chapter 2

General Materials and Methods

2.1 Animals

2.1.1 Adult sheep in the terminal stages of OPA

The acquisition of animals for research into ovine pulmonary adenocarcinoma (OPA) in adult field cases depends entirely on the goodwill of sheep farmers, who have agreed with the Moredun Research Institute to supply sheep suspected of having OPA. If symptoms of OPA, such as laboured breathing, weight loss and/or the production of lung fluid were identified by the farmer, the veterinary staff at the Institute were notified, and the animal was collected from the farm immediately. On arrival at the Moredun Research Institute a full veterinary examination was performed to determine the health of the sheep. The severity of symptoms in OPA cases ranged from slightly laboured breathing with limited weight loss to severe emaciation and breathing difficulties. The majority of sheep involved in this study had progressed into the terminal stages of OPA, with severe dyspnea, and in many cases, the production of lung fluid.

All suspected OPA cases at the Moredun Research Institute were housed in pens separated from other sheep, and all contact with other animals was not permitted. Lung fluid if produced, and blood samples were collected (section 2.1.3.1 and 2.1.4, respectively). During this project twelve cases of OPA were confirmed in adult sheep, aged between three and five years old, but on one occasion OPA was diagnosed in a female yearling. The majority of OPA cases were ewes, with several breeds of sheep implicated including Greyface, Texel and Blackface-X-Blackface Leicester. The male and female adult sheep used as control animals in these studies, were aged one to four years, and from various breeds including Dorset and Suffolk-X. All the controls were from stock at the Moredun Research Institute.

The animals were humanely killed by an overdose of pentobarbitone (Rhône Merieux, Tallaght, Dublin) and post mortem examinations performed to determine the disease status of each animal. The thoracic lymph nodes were removed, and the mediastinal and bronchial lymph nodes examined macroscopically for any evidence of enlargement. In the lungs, the presence of neoplastic lesions, identified as a large tumour mass and/or discrete nodules was recorded, together with any observed secondary infections. Samples of lung, tumour, mediastinal and bronchial lymph node collected at necropsy were fixed in formaldehyde and paraformaldehyde for histological analysis. Identification of tumour as isolated and/or confluent pulmonary neoplastic lesions histologically confirmed the presence of OPA. In some cases, hyperplasia of bronchial associated lymphoid tissue, mononuclear inflammation, and an influx of enlarged alveolar macrophages and neutrophils to the site of the tumour, were detected. Secondary infections have also been identified in association with OPA, predominately desquamative or suppurative pneumonia, and/or bacterial infections such as *Pasteurella* species. Histological analysis was performed at the Moredun Research Institute by Dr. Lorenzo Gonzalez (Veterinary Laboratory Agencies, Penicuik, Midlothian. Scotland).

2.1.2 Experimental JSRV infection of neonatal lambs

To monitor immune responses during the pre-clinical stages of disease, two experiments were performed. All lambs were born and housed at the Moredun Research Institute.

- i) Conventionally Housed Lambs: Ten Dorset lambs, born under natural conditions were used in this study. The lambs were fed by their mothers, consequently they received colostrum. From four weeks old their diet was

supplemented with high protein proprietary weaner pellets. They continued to be reared by their mothers until eight weeks of age. From fourteen weeks, the lambs were fed rearer pellets (lamb nuts), with the composition of lipids, protein and fibre modified to meet their dietary requirements. The lambs were fed using the lamb creep system (a feeding mechanism, which allows only lambs access to the feed whilst restricting the adults). A supply of hay was also available. The lambs remained on this diet until the termination of the experiment. At seven days old, six lambs (3 sets of twins) were intratracheally inoculated with JSRV, purified from lung fluid (section 2.1.3.2). The four remaining lambs (two sets of twins) were used as un-inoculated control animals. All animals were housed identically, with the JSRV-infected lambs and their mothers, the control lambs together with their mothers, kept in separate pens.

ii) Specific pathogen free lambs (SPF): To extend the experiment performed with the conventionally housed lambs and minimise exposure to outside influences and secondary infections, the above experiment was repeated with ten Suffolk-X lambs. At four days old, six lambs (two sets of twins and two single lambs) were intratracheally inoculated with JSRV, purified from lung fluid (section 2.1.3.2). The four remaining lambs (two sets of twins) acted as un-inoculated control animals throughout the experiment.

The SPF lambs were born by caesarean section and aseptically hand reared under modified conditions with sterilised air supply, bedding and food. Although not sterile, SPF containment supports a controlled environment, minimising exposure to infectious agents, to which lambs would otherwise be susceptible. From birth the lambs were given UHT cow milk. At two weeks old, this was

supplemented with Locush™ lamb feed until the introduction of supplemented feed at four weeks, using the lamb creep system outlined in section 2.1.2(i). The volume of UHT milk given was reduced until the lambs were fully weaned at six weeks. At twelve weeks the lambs were introduced to rearer pellets (lamb nuts), which they received thereafter, until the end of the experimental period. Sterilized hay was also available.

2.1.3 JSRV inoculation of experimental lambs

2.1.3.1 Lung fluid in OPA sheep

Lung fluid, identified as a nasal exudate in OPA cases, was collected by ‘the wheelbarrow technique’ where the hind-quarters of the sheep were raised whilst holding down the head, allowing the fluid to drain into a sterile collection vessel (Chapter 1, section 1.5.1).

2.1.3.2 Preparation of JSRV inoculum

The inoculum used to induce experimental infection in both the conventionally housed and SPF neonatal lambs was partially purified JSRV from lung fluid, collected from OPA cases. The JSRV inoculum was prepared by Patricia Dewar (Moredun Research Institute), according to the method originally described by Sharp *et al* (1983). The lung fluid was clarified by filtering through a sterile gauze-covered-funnel to remove large particles. The filtrate was then centrifuged for 30 minutes at 1080g at 4°C. The clarified lung fluid was layered on a 3ml/3ml, 50/25% glycerol-TNE (1M sodium chloride, 0.1M Trisma-base, 10mM EDTA) buffered cushion, in a Beckman SW28 centrifuge tube, using a 16gauge hypodermic syringe. JSRV was concentrated through the cushion by centrifugation at 100,000g for 60 minutes at 4°C. The supernatant was removed by aspiration and the tubes

inverted onto tissue paper to drain for 2-5 minutes. Residual fluid was removed from the tube with a tissue. The pellet was re-suspended in PBS or supernatant to give a X15 final concentration with respect to the starting volume. The inoculum administered to the conventionally house lambs comprised JSRV re-suspended in PBS, the SPF lambs were inoculated with JSRV re-suspended in the supernatant. The inocula were stored at -70°C until required and 5ml X15 JSRV/animal was administered by intratracheal inoculation. All the control animals remained uninoculated.

2.1.4 Blood sample collection

For all animals, blood samples were collected by jugular venepuncture into sterile Vacutainer Brand™ evacuated blood collection tubes (Becton Dickinson, Rutherford, NJ, USA) containing preservative-free heparin [10 units per ml blood] (Sigma, Poole, Dorset), and 100µl aliquots were removed from each sample, prior to PBMC purification, and retained for the neutrophil counts (section 2.4).

2.1.5 Diagnosis of disease

In all experiments, when clinical signs of OPA were apparent, the lambs were humanely killed and post mortem examinations performed as described in section 2.1. Identification of lesions as small discrete nodules at isolated foci and/or confluent pulmonary tumour macroscopically and histologically confirmed the presence of OPA. Histological investigations were performed at the Moredun Research Institute by Dr. Lorenzo Gonzalez (Veterinary Laboratory Agencies, Penicuik, Midlothian. Scotland).

In some cases tissue samples were collected into a cryomatrix resin (Thermo Shandon, Pittsburgh PA, USA), snap frozen in liquid nitrogen and stored at -70°C ,

or fixed in paraformaldehyde, formaldehyde or zinc salts fixative (ZSF) and paraffin embedded. The tissue samples were then prepared for immunohistochemical analysis, full details of these procedures are in section 2.10, 2.11, 2.12, 2.13 and Chapter 5.

2.2 Purification of peripheral blood mononuclear cells (PBMC)

Neutrophilia in the peripheral blood of adult sheep in the terminal stages of OPA is a recognised phenomenon (Rosadio and Sharp 1992, Holland *et al* 1999). Inaccurate data would be generated during comparative studies by inadequate depletion of neutrophils from the blood. For example, to measure the proliferative responses of infected and control animals, identical concentrations of PBMC from both groups are required. A high numbers of neutrophils retained in the PBMC samples from infected animals would limit the number of lymphocytes available for stimulation.

2.2.1 Purification of PBMC and depletion of neutrophils

The large population of neutrophils present in the blood of adult OPA cases was not adequately removed by the standard laboratory PBMC purification method (Boyam 1968), where the whole blood is diluted or layered directly onto a density gradient. Therefore to purify the PBMC and to deplete the neutrophils three methods were tested.

- i) Removal of neutrophils by centrifugation of whole blood prior to PBMC purification: Samples of 10ml whole blood were transferred to conical tubes and centrifuged at 300g for 15 minutes. The buffy coat, together with a small volume of plasma was recovered and re-suspended in 1ml RPMI-1640

medium (Gibco BRL, Paisley, Renfrewshire) containing 5µg/ml glutamine, 5µg/ml penicillin/streptomycin, 0.5µg/ml gentamicin, 5µg/ml fungizone (Sigma, Poole, Dorset) and 10units/ml heparin. The sample was very gently mixed. *The cell suspension was then layered over 5ml Lymphoprep™ (Nycomed Pharma AS, Oslo, Norway) and centrifuged at 300g for 15 minutes. The interface cells were recovered and re-suspended in 10ml RPMI-1640 containing 10% heat inactivated foetal calf serum (Gibco BRL, Paisley, Renfrewshire) and 10units/ml heparin. The samples were centrifuged at 240g for 10 minutes and the supernatant decanted. The cell pellet was then washed twice by centrifugation at 240g for 5 minutes and re-suspended in 5ml RPMI-1640 medium (no added heparin). After a final wash the supernatant was decanted and the cells re-suspended in 5ml RPMI-1640 with 10% heat inactivated foetal calf serum added. Cytospins of the purified PBMC microscopically showed that few neutrophils were retained. Furthermore, this method did not adversely affect the number of PBMC recovered. The procedure was cost effective as no other reagents were required and only an extra 15 minutes centrifugation per sample was necessary.

- ii) Sedimentation. Using citrate as an anticoagulant, fresh venous blood was collected and centrifuged at 180g for 20 minutes. The cell pellet was re-suspended in four volumes of 6% (w/v) dextran T500: 0.9% (w/v) NaCl. An equal volume of phosphate buffered saline (PBS) was added and the cells left to sediment for 25-40 minutes at room temperature. The leukocyte rich upper layer was collected. The PBMC were then purified as detailed from * in

method (i). As the cells were left to sediment for up to 40 minutes, this method was time consuming and additional reagents were required. The resulting PBMC were not purified to a high standard as microscopic observation of the PBMC cytopins revealed many neutrophils were still present (data not shown).

- iii) An adaptation of the method developed by Carlson and Kaneko (1972) to isolate leukocytes from bovine peripheral blood was tested. Briefly 10ml whole blood was centrifuged at 1000g for 15 minutes. The buffy coat and uppermost portion of the red cell column was recovered into 4ml 0.08% NaCl in 13.2mM PBS (pH 6.8). Erythrocytes in the suspension were lysed by the addition of 20ml deionised water and gently mixed for 30 seconds. The isotonicity was then restored by the addition of 10ml PBS (13.2mM pH 6.8) containing 2.7% NaCl. The cells were washed a further three times by centrifugation at 200g for 10 minutes and re-suspended in 5ml PBS. Additional reagents were required for this method, and although the majority of neutrophils were removed (detected microscopically), this method also resulted in a reduced number of purified PBMC recovered (data not shown).

All three methods to purify the PBMC were tested on three separate occasions, twice with blood from adult OPA cases and once with an uninfected control sheep. The results obtained from the infected animals were consistent with those detailed above, and no detrimental effects were observed in the PBMC from the control animal.

Therefore method (i) was selected as the PBMC purification protocol. Consequently, to ensure that consistency was maintained with all blood samples, this method was

employed not only with adult OPA cases, but also with JSRV-infected lambs and all control animals.

2.2.2 Cell counts

A standard haematological counting fluid, 1.5% acetic acid, 0.01% Giemsa stain (BDH, Poole, Dorset) in distilled water was used to dilute the cells and lyse any remaining red blood cells. A suspension of 10 μ l PBMC was added to 90 μ l counting fluid and gently mixed. The cells were counted using a Neubaur haemocytometer and adjusted to a final concentration of 10⁶ PBMC/ml.

2.3 Phenotyping

2.3.1 Phenotyping of purified PBMC

2.3.1.1 Immunolabelling of PBMC

PBMC phenotyping by indirect immunofluorescence was performed using standard laboratory procedures (Bachh *et al* 1995, Lloyd *et al* 1995). The purified PBMC were adjusted to a final concentration of 10⁶ PBMC/ml. The PBMC at 5 \times 10⁵ PBMC/ml were immunolabelled with a non-specific mouse immunoglobulin (IgG) as a control, (initially bovine serum albumin (BSA) was used to identify non-specific fluorescence), and mouse anti-sheep monoclonal antibodies specific for CD2⁺ T cells (135A), $\gamma\delta$ T cells (86D) (Mackay *et al* 1988), CD4⁺ T cells (17D), CD8⁺ T cells (SBU-T8) (Maddox *et al* 1985), B cells (α -light chain VPM8) (Yirrell *et al* 1991) and MHC class II antigens (VPM54) (Gupta *et al* 1986), (all supplied by Prof. John Hopkins, University of Edinburgh). The immunolabelled samples were incubated for 30 minutes at room temperature, in the dark. After incubation, 1ml PBS was added to each sample and the tubes centrifuged at 240g for 5 minutes. The supernatant was decanted and the secondary antibody, a rabbit anti-mouse Ig/fluorescein (FITC)

conjugate (DAKO, Ely, Cambridgeshire) was added at 100µl volume of 1:50 dilution per sample. The samples were incubated for 30 minutes at room temperature, in the dark. After incubation, 1ml PBS was added and the samples centrifuged at 300g for 5 minutes. The supernatant was decanted and 1ml PBS and 100µl 10% formol saline added. The samples were stored at 4⁰C for a maximum of 56 hours before analysis.

2.3.1.2 Flow cytometry

The results were determined by flow cytometry using an EPICS XL-MCL™ profile (Coulter Electronics, Miami, Florida, USA), utilizing an argon ion laser set at a wavelength of 488nm. The PBMC were separated from the cell debris, characterised by forward scatter (FS) determining cell size, and side scatter (SS) determining cellular granularity. The immunolabelled lymphocytes were separated from the PBMC population with gates set around the lymphocyte region on 90⁰ and forward angle scatter (Figure 2.1). Fluorescence data were collected on a minimum of 2000 lymphocytes, with a 1% background control. An example of the immunolabelled phenotypic profile, detected in adult sheep peripheral blood is shown in Figure 2.2.

2.3.1.3 The optimisation of monoclonal antibody titres

The affinity of individual monoclonal antibodies for cell surface receptors varies extensively. Therefore to analyse the leukocyte phenotypic profile of the blood, the optimum titre for each monoclonal antibody was determined from a titration series (Figure 2.3). The PBMC were immunolabelled as described in section 2.3.1.1 with BSA as a control sample and 1µl, 2.5µl, 5µl, 10µl and 20µl monoclonal antibody/5 x 10⁵ PBMC. At a concentration of 1µl/5 x 10⁵ PBMC the percentage of

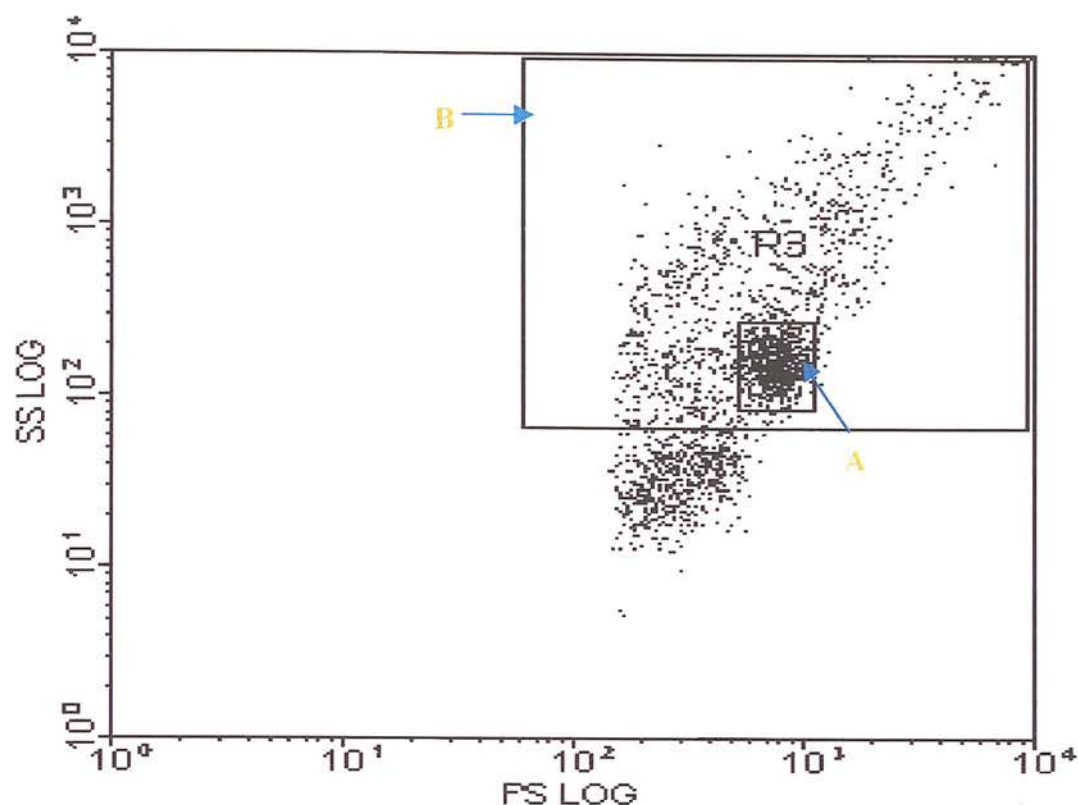


Figure 2.1 Computer generated dot plot of sheep peripheral blood showing scatter profile of the purified PBMC. The purified PBMC were immunolabelled with mouse anti-sheep monoclonal antibodies specific for CD2 T cells, B cells and MHC class II antigens. The results were determined by flow cytometry. *Vertical axis:* side scatter (SS log) defining cellular granularity, *horizontal axis:* forward scatter (FS log) defining cell size. The results show the scatter profile of purified PBMC with defined lymphocyte region (A), identified by positively immunolabelled CD2 T cells and B cells. Cells expressing MHC class II antigens were identified by gating the entire PBMC population (B), incorporating the lymphocyte region and other cells not recognised by the panel of monoclonal antibodies used, but excluding the cell debris.

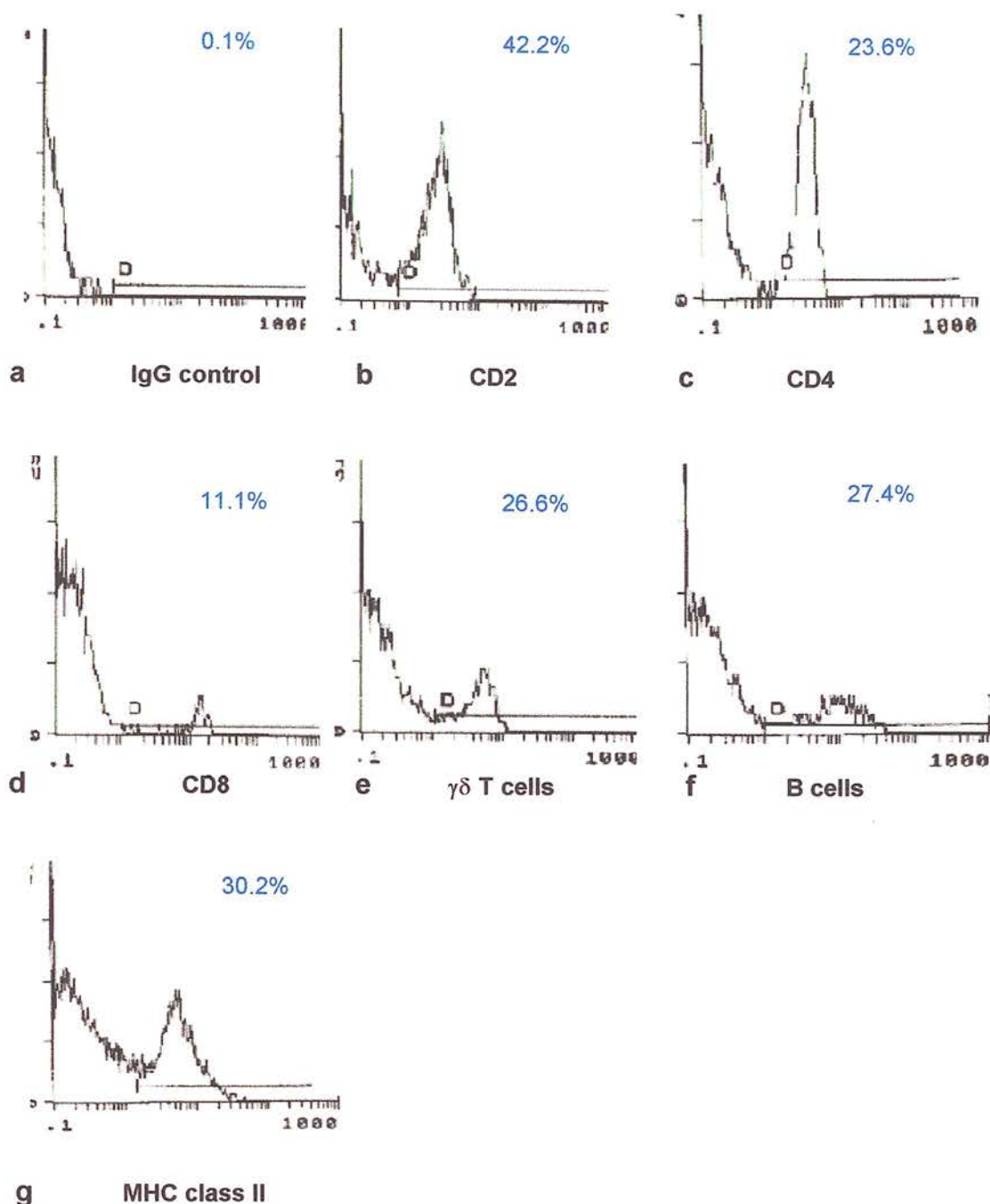


Figure 2.2 Histograms of lymphocyte subsets and MHC class II expression in the purified PBMC of sheep. The PBMC were immunolabelled with monoclonal antibodies specific for CD2, CD4, CD8, $\gamma\delta$ T cells, B cells and MHC class II antigens, and the percentage of positively immunolabelled cells detected by flow cytometry, Y axis: cell frequency. X axis: log green fluorescence. Peaks at origin were due fluorescence of background debris (Figure 2.2a). Figures 2.2 b-f: Lymphocyte subsets were selected by gating, based on light scatter characteristics determining the lymphocyte region. Figure 2.2g: Percentage of cells expressing MHC class II antigens was selected by gating entire PBMC population, excluding debris.

positive cells was comparable with the background control. At $2.5\mu\text{l}/5\times 10^5$ PBMC an increase in the percentage of each subset was evident but increased further with the addition of $5\mu\text{l}/5\times 10^5$ PBMC. No significant difference in the percentage of positive cells was detected when labelled with $5\mu\text{l}$, $10\mu\text{l}$, and $20\mu\text{l}/5\times 10^5$ PBMC. Consequently, the optimum titre for all the monoclonal antibodies was $5\mu\text{l}/5\times 10^5$ PBMC.

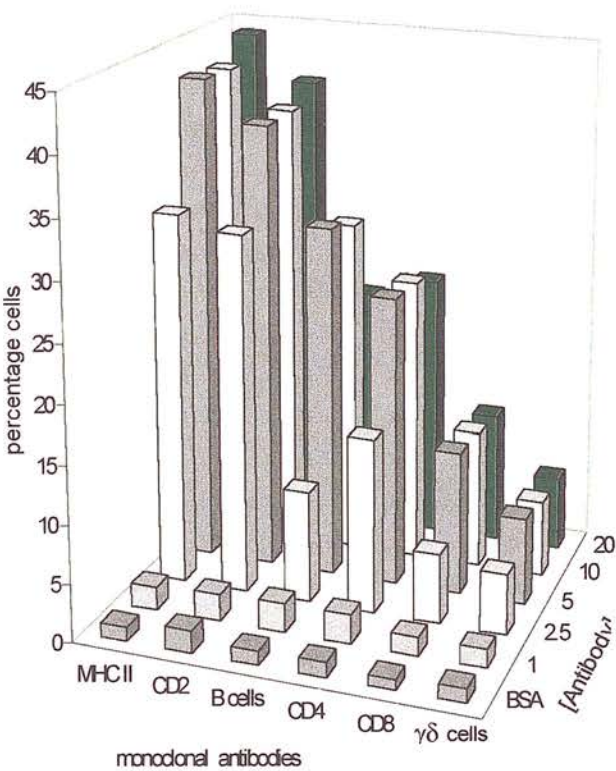


Figure 2.3 Optimum monoclonal antibody concentration. The PBMC subsets were immunolabelled with a titration series of mouse anti-sheep monoclonal antibodies, and the results were determined by flow cytometry. Vertical axis: percentage of positive labelled cells in sheep peripheral blood. 1st horizontal axis: panel of monoclonal antibodies. 2nd horizontal axis: monoclonal antibody concentration ($\mu\text{l}/5\times 10^5$ PBMC). The results for the lymphocyte subsets are shown as percentage positive immunolabelled cells/lymphocyte region. Cells immunolabelled for MHC class II antigens are shown as percentage positive immunolabelled cells/PBMC.

2.3.2 Phenotyping using whole blood samples

2.3.2.1 Identification of the lymphocyte region in whole blood samples

The purification of peripheral blood mononuclear cells (PBMC) from a large number of samples is a labour intensive and time-consuming procedure, with the condition of the PBMC deteriorating if they are retained on the bench for long

periods of time. To expedite laboratory procedures, phenotyping using whole blood was considered. It was essential to ensure that this procedure could be performed consistently; not only with blood from uninfected animals, but that the presence of JSRV did not in any way compromise the assay. Therefore the phenotypic profiles of mononuclear cells present in whole blood samples were compared with those in the corresponding purified PBMC.

Aliquots of 100 μ l whole blood were immunolabelled by incubation with monoclonal antibodies following the protocol for PBMC phenotyping described in section 2.3.1.1. After incubation with the secondary antibody, and prior to the addition of PBS and formol saline, the red blood cells were lysed by adding 2ml ErthyrolyseTM red blood cell lysis buffer (Serotec, Kidlington, Oxford) diluted 1:10 in distilled water. The samples were incubated in the dark for 10 minutes at room temperature. After incubation 2ml PBS was added and the samples centrifuged at 300g for 5 minutes. The supernatant was decanted and the cells re-suspended in 1ml PBS, centrifuged at 300g for 5 minutes, the supernatant decanted, and 1ml PBS and 10% formol saline added. PBMC were purified from the remainder of the corresponding whole blood sample (section 2.2.1) and immunolabelled as detailed in section 2.3.1.1. The lymphocyte subset frequencies identified in whole blood samples and corresponding PBMC were determined and compared by flow cytometry as detailed in section 2.3.1.2.

The large amount of cell debris, and the presence of the polymorphonuclear cells retained in the whole blood impeded identification of the lymphocyte population (Figure 2.4a). Therefore, the lymphocyte region was first identified and gated in the corresponding purified PBMC sample as detailed in section 2.3.2 using

the CD2⁺ monoclonal antibody (Figure 2.4b). The lymphocyte region parameters were confirmed using a B cell monoclonal antibody. After gating, and retaining the parameters the whole blood samples could be analysed.

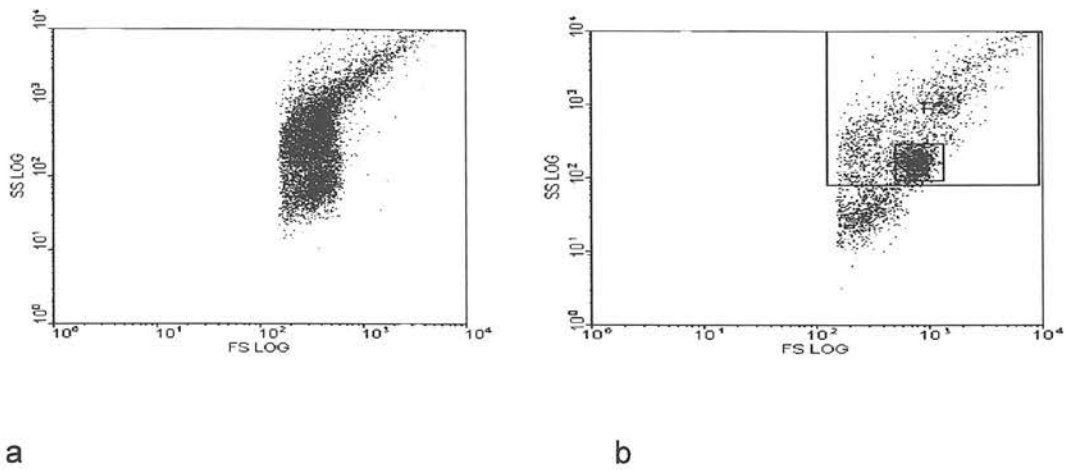


Figure 2.4 Scatter profile of mononuclear cells from whole blood and purified PBMC. Computer generated dot plot of sheep peripheral blood. *Vertical axis:* side scatter (SS log) defining cellular granularity, *horizontal axis:* forward scatter (FS log) defining cell size. The results show the scatter profile of whole blood sample (Figure 2.4a), with large amounts of cell debris and polymorphonuclear cells obscuring the lymphocyte regions. Figure 2.4b. shows the scatter profile of purified PBMC with defined lymphocyte region (a), identified by CD2⁺ and B cell monoclonal antibody labelling. Cells expressing MHC class II antigens were identified by gating the entire PBMC population (b), incorporating the lymphocyte region and other cells not recognised by the panel of monoclonal antibodies used, but excluding the cell debris.

2.3.2.2 Whole blood and PBMC phenotypic comparison

The percentage of lymphocyte subsets identified within whole blood samples was compared with the percentage present in the corresponding purified PBMC from the same adult sheep. Purified PBMC and mononuclear cells present in whole blood samples were phenotyped using monoclonal antibodies recognising CD2, CD4, CD8 and $\gamma\delta$ T cells and B cells, and the results determined by flow cytometry (sections 2.3.1 and 2.3.2, respectively). The percentage of positive immunolabelled

lymphocytes were decreased in the whole blood samples in comparison with the percentage detected in the purified PBMC. The phenotypic profile from three adult OPA cases, (Figure 2.5a) revealed significantly ($p = 0.02$) reduced percentages of CD2 T cells (PBMC 31.3 ± 4.6 , whole blood 11.1 ± 2.7), $\gamma\delta$ T cells (PBMC 12.8 ± 0.4 , whole blood 4.9 ± 0.9 ($p = 0.001$)) and B cells (PBMC 31.3 ± 4.4 , whole blood 19.2 ± 1.8 ($p = 0.02$)). The phenotypic profile from the whole blood and the purified PBMC of three uninfected adult sheep is shown in Figure 2.5b. Comparison between PBMC and whole blood detected significantly ($p = 0.003$) reduced percentages of CD2 T cells (PBMC 49.5 ± 0.4 , whole blood 15.6 ± 5.1) and $\gamma\delta$ T cells (PBMC 11.6 ± 2.1 , whole blood 4.3 ± 1.5 ($p = 0.04$)). No significant difference in the percentage of positively immunolabelled CD4 or CD8 T cells was identified between the whole blood and purified PBMC, from either group of animals.

The decrease in CD2 and $\gamma\delta$ T cells identified in the whole blood may suggest that the epitopes present on the cell surface were masked by the large accumulation of debris present in the sample. The decreased B cell population observed in the whole blood from the OPA sheep, whilst not evident in the control animals, was not suggestive of a JSRV-related response as no difference was detected in the corresponding purified PBMC sample. The percentages of immunolabelled lymphocyte subsets, detected in the purified PBMC of the control sheep, were comparable to those previously identified in other studies characterising the ovine PBMC phenotypic profile (Smith *et al* 1994, Bachh *et al* 1995). However overall, the inconsistencies identified with the whole blood and purified PBMC suggest that whole blood phenotyping was not the optimal method for comparative studies during JSRV infection and the development of OPA. To ensure constant

reliability during all experiments and monitoring programmes throughout this project, only purified PBMC were used.

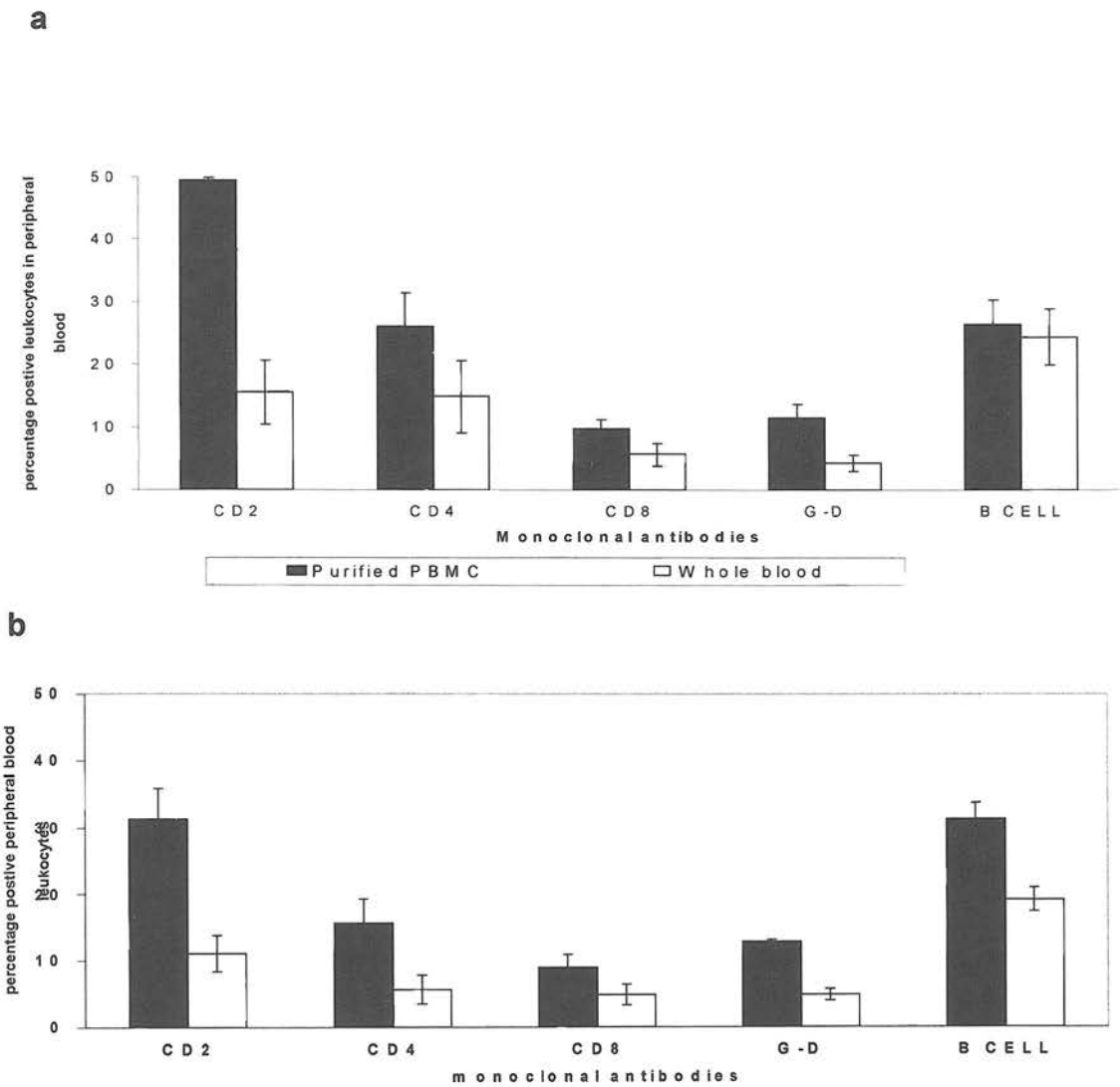


Figure 2.5 Phenotyping mononuclear cells from whole blood and purified PBMC.

Comparison of the lymphocyte phenotypic profile of purified PBMC and whole blood from 3 adult OPA cases (Figure 2.5a) and 3 uninfected adult sheep (Figure 2.5b). Results are shown as percentage positive immunolabelled cells in peripheral blood, \pm SEM.

2.4 Determination of neutrophil numbers

The neutrophils present in the blood samples were determined from 100 μ l aliquots of whole blood retained prior to the purification of the PBMC. The red blood cells were lysed by adding 2ml ErthyrolyseTM red blood cell lysis buffer diluted 1:10 in distilled water to the whole blood. The samples were incubated for 10 minutes at room temperature. The cells were washed by adding 2ml PBS per sample and centrifuged at 300g for 5 minutes, the supernatant was decanted, and the cells re-suspended in 1ml PBS, centrifuged at 300g for 5 minutes, the supernatant decanted, and 1ml PBS and 10% formol saline added.

Initially, the proportion of neutrophils present in each blood sample was determined microscopically based on cellular morphology. To ascertain the presence of neutrophils in the samples 200 μ l aliquots were removed and cytopins prepared (Cytocentrifuge, Shandon Southern Products Ltd., Runcorn, Cheshire) following the manufacturer's instructions. The cells were fixed in methanol for 10 minutes, air-dried and stained for 15 minutes with 1:10 Giemsa stain (BDH, Poole, Dorset), gently washed under running tap water and air-dried.

In subsequent studies the percentage of neutrophils present in the whole blood was ascertained by flow cytometry (EPICS XL-MCLTM) profile, based on cell size and granularity, determined by forward and side scatter profile, utilizing an argon ion laser set at a wavelength of 488nm. The neutrophils present in the purified PBMC sample (Figure 2.6a) were gated on 90⁰ and forward angle scatter and the set parameters were used to identify the neutrophils present in the whole blood sample (Figure 2.6b). Data were collected on a minimum of 5000 events.

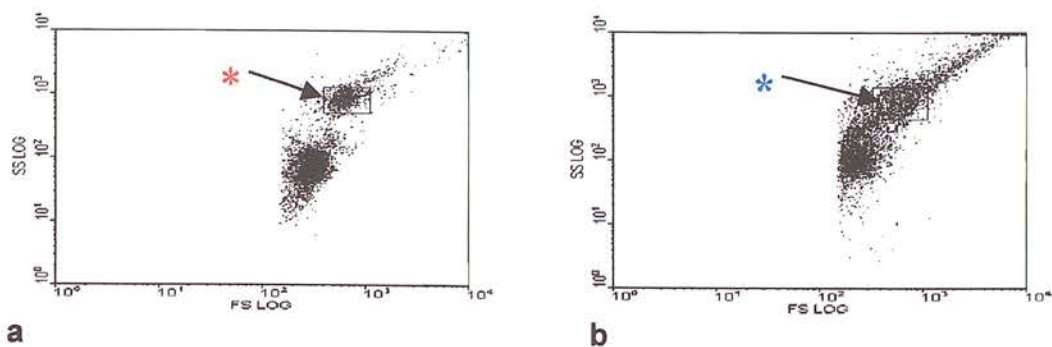


Figure 2.6 Neutrophils detected in sheep peripheral blood by flow cytometry. Computer generated dot plots of sheep peripheral blood. *Vertical axis:* side scatter (SS log) defining cellular granularity, *horizontal axis:* forward scatter (FS log) defining cell size. Figure 2.6a: Scatter profile of purified PBMC showing *gated neutrophils. These parameters were set and retained to gate the neutrophil population present in the whole blood sample. Figure 2.6b: The scatter profile of the corresponding whole blood sample with the *gated neutrophil population identified.

2.5 Lymphoproliferation

2.5.1 Lymphoproliferation of purified PBMC

The level of lymphoproliferative response to mitogen stimulation is species specific, and the concentration of individual mitogens required to stimulate optimum responses differs considerably. Therefore the optimum titre for each mitogen used in lymphoproliferation studies was identified from a concentration curve.

The *in vitro* lymphoproliferative responses to mitogens were measured as previously described (Burrells and Wells 1977, Wells *et al* 1979, Kilpatrick 1998). The PBMC were purified from the venous blood of sheep as described in section 2.2.1 and adjusted to a final concentration of 10^6 PBMC/ml (section 2.2.2). Various concentrations of phytohaemagglutinin (PHA), concanavalin A (ConA) and pokeweed mitogen (PWM) (all from Sigma, Poole, Dorset) were added to 10^6 PBMC/ml RPMI-1640 medium. Control samples (excluding a lectin) were prepared also. All samples, at 2×10^5 PBMC/well were dispensed, in triplicate, into sterile 96-

well, round bottomed plates (Bibby Sterilin, Stone, Staffordshire). The plates were incubated in 5% CO₂ at 37°C for a total of 96 hours, with the addition of 0.7μCi/well [*methyl*-³H]thymidine (specific activity 2.0Ci/mmol, Amersham, Little Chalfont, Buckinghamshire) for the final 16 hours. The cells were then harvested onto fibreglass sheets (Automash 2000, Dynatech), air-dried and transferred to vials. Commercial liquid scintillant (Packard Bioscience, Groningen, The Netherlands) at 1ml per vial was added and ³H-thymidine uptake measured as counts per minute (cpm) (Packard Tri-Carb Liquid Scintillation Analyser). The optimum concentrations, identified by the maximum response, were found to be 12μg/ml for PHA, 15μg/ml for ConA and 2μg/ml for PWM. (Figure 2.7 a, b and c.).

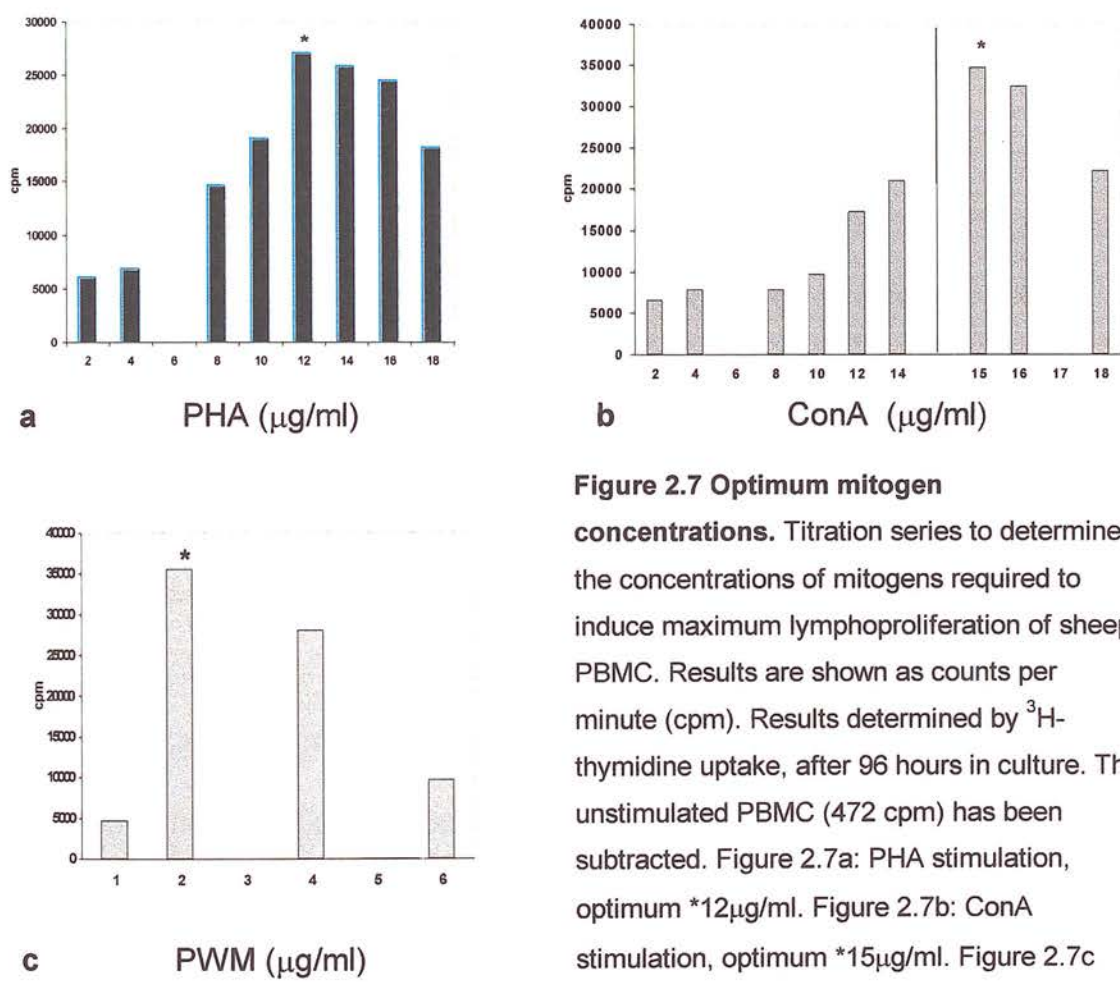


Figure 2.7 Optimum mitogen concentrations. Titration series to determine the concentrations of mitogens required to induce maximum lymphoproliferation of sheep PBMC. Results are shown as counts per minute (cpm). Results determined by ³H-thymidine uptake, after 96 hours in culture. The unstimulated PBMC (472 cpm) has been subtracted. Figure 2.7a: PHA stimulation, optimum *12μg/ml. Figure 2.7b: ConA stimulation, optimum *15μg/ml. Figure 2.7c PWM stimulation, optimum *2ug/ml.

2.5.2 Lymphoproliferation of PBMC depleted of adherent cells

A 20ml volume of venous blood was collected (section 2.1.4), and the PBMC were purified as described in section 2.2.1(i)). The cells were counted (section 2.2.2) and adjusted to a final concentration of 10^7 cells/ml RPMI-1640 medium, containing antibiotics and 10% foetal calf serum. Three aliquots of the PBMC were prepared. Entire-PBMC Lymphoproliferation was performed with 10^6 PBMC/ml and the optimum titre of the required mitogen (including a control sample, without any mitogen), as detailed in section 2.5.1. An aliquot of 5×10^5 cells was retained for detection of MHC class II antigens by flow cytometry.

PBMC depleted of adherent cells The adherent cells were removed from the PBMC prior to lymphoproliferation by incubating a 5ml volume of 10^7 PBMC/ml in a 50ml tissue culture flask in 5% CO₂ at 37°C for 1 hour. The non-adherent cells were decanted, and a 100µl aliquot removed and retained for cytospin analysis. The cells were counted (section 2.2.2) and 5×10^5 cells retained for detection of MHC class II antigens by flow cytometry. The optimum titre of the required mitogen was added to a concentration of 10^6 cells/ml and lymphoproliferation (including a control sample, without any mitogen) was then performed as detailed in section 2.5.1. The adherent cells were scraped from the flask using a sterile cell scraper (Merck Eurolab Ltd, Lutterworth, Leic.) and re-suspended in 1ml RPMI-1640 medium, containing antibiotics and 10% foetal calf serum. A 100µl aliquot of cells was removed and retained for cytospin analysis.

Reconstituted-PBMC The adherent cells were centrifuged at 300g for 5 minutes and the supernatant decanted. The pellet of adherent cells was restored to the remaining depleted PBMC, the cells were counted (section 2.2.2) and adjusted to a final

concentration of 10^6 PBMC/ml RPMI-1640 medium, containing antibiotics and 10% foetal calf serum. A 100 μ l aliquot was removed and retained for cytopsin analysis, and 5×10^5 PBMC were retained for detection of MHC class II antigens by flow cytometry. Lymphoproliferation assays (including a control sample, without any mitogen) were then performed as detailed in section 2.5.1.

2.5.3 Confirmation of removal of adherent cells from the PBMC

From the retained aliquots, cytopsin of the PBMC depleted of adherent cells, the isolated adherent cells and the reconstituted-PBMC were prepared as detailed in section 2.4. Microscopic examination of the cellular morphology confirmed that the adherent cells had been removed from the PBMC, as only lymphocytes were identified in the depleted PBMC sample. In the adherent cell sample no lymphocytes were evident, with only monocytes identified. The reconstituted-PBMC sample was found to contain a mixed population of lymphocytes and monocytes (results not shown). To confirm that the expression of MHC class II was reduced in the adherent-cell-depleted-PBMC, compared with the entire and the reconstituted-PBMC, the cells were immunolabelled with a monoclonal antibody specific for MHC class II antigens (and IgG control), following the protocol detailed in section 2.3.1.1. The results, determined by flow cytometry (section 2.3.1.2) are shown in Figure 2.8, confirmed that the percentage of immunolabelled cells expressing MHC class II antigens was reduced in the PBMC depleted of adherent cells (9.0%) in comparison with the percentages detected in the entire PBMC (39.8%) and reconstituted PBMC (35.4%).

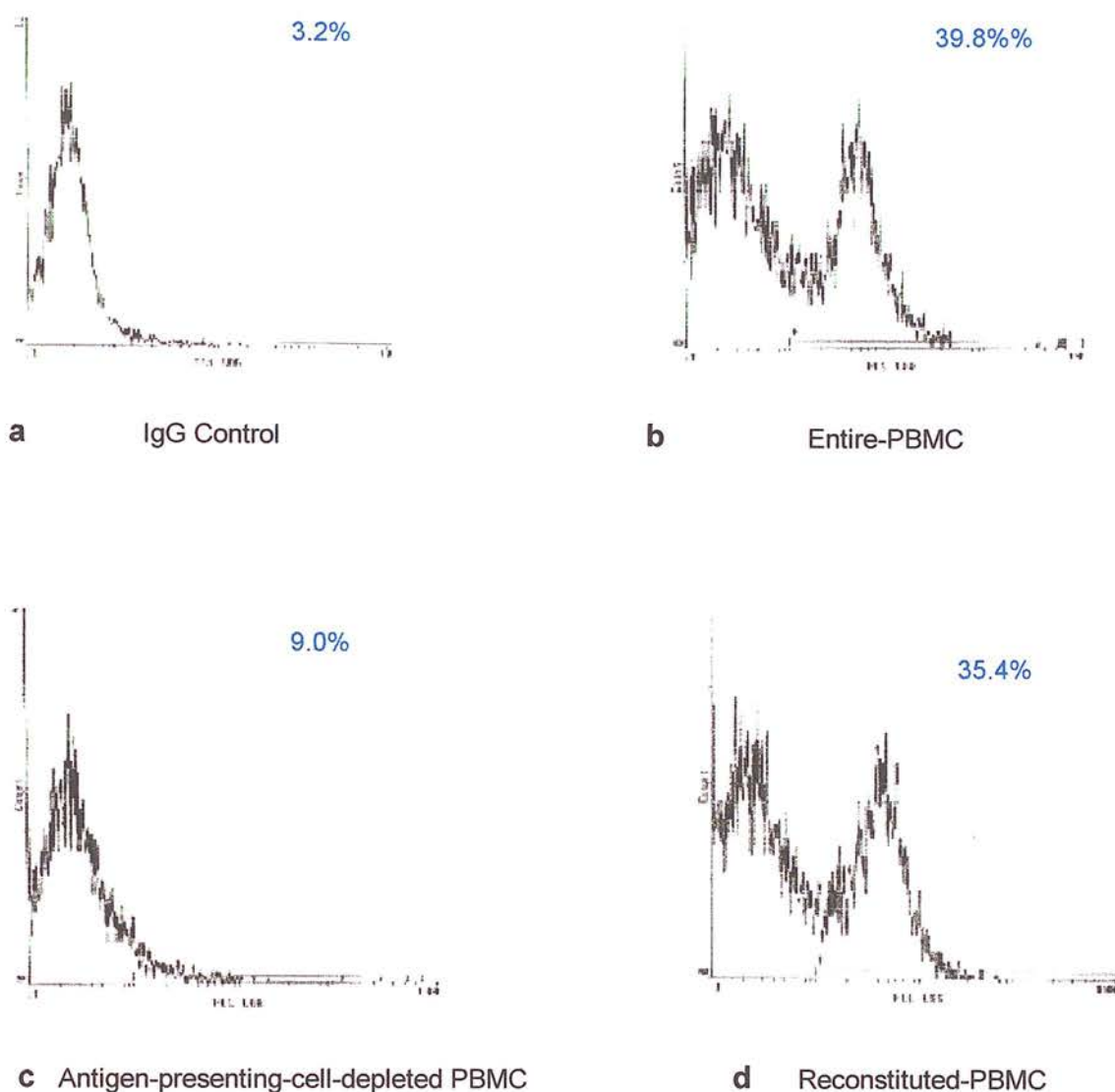


Figure 2.8 Flow cytometry histograms of MHC class II detected in the entire-PBMC, PBMC depleted of adherent cells and reconstituted-PBMC. The percentage of cells expressing MHC class II antigens in the entire-PBMC, PBMC depleted of adherent cells and reconstituted-PBMC (adherent cells replaced) were compared. The cells were immunolabelled with a mouse ant-sheep monoclonal antibody specific for MHC class II antigens and the results determined by flow cytometry. The results are shown as percentage positive MHC class II immunolabelled cells detected in each sample. Figure 2.8a: The IgG control sample (entire-PBMC) with a 3.2% background autofluorescence. Figure 2.8b: The entire-PBMC. Figure 2.8c: The percentage of MHC class II expression in the PBMC depleted of adherent cells. Figure 2.8d: The reconstituted-PBMC (after the adherent cells had been returned to the PBMC).

2.6 Storage conditions of samples for *in vitro* studies

2.6.1 The effects of storage on venous blood samples

For comparative analysis, consistency of the PBMC purified from each blood sample must be maintained. To ascertain the optimum conditions for holding whole blood, without degradation of the cell surface antigens, the effects of time and temperature on whole blood, prior to the purification of PBMC for phenotyping analysis and lymphoproliferation assays were examined. The phenotypic profile of stored immunolabelled PBMC was evaluated also.

2.6.1.1 Effect of whole blood storage on the phenotypic profile of the PBMC

The PBMC, purified from freshly collected blood samples and after the heparinised blood had been stored for 24 and 48 hours at room temperature and 4⁰C, were phenotyped using monoclonal antibodies specific for CD2, CD4, CD8, $\gamma\delta$ T cells and B cells (section 2.3.1.1) and the results were determined by flow cytometry (section 2.3.1.2).

As shown in Figure 2.9, storage of heparinised whole blood at room temperature, for up to 48 hours did not adversely affect the phenotypic profile of the subsequently purified PBMC subsets, as the results were comparable to those obtained with fresh blood and are in accordance with the finding of Lloyd *et al* (1995). In contrast blood stored at 4⁰C for 24 and 48 hours resulted in reduced lymphocyte sub-population detected, with all subsets affected. Therefore it was not possible to store the blood samples at 4⁰C, even for a period of 24 hours, as this adversely affected the quality of the subsequently purified PBMC.

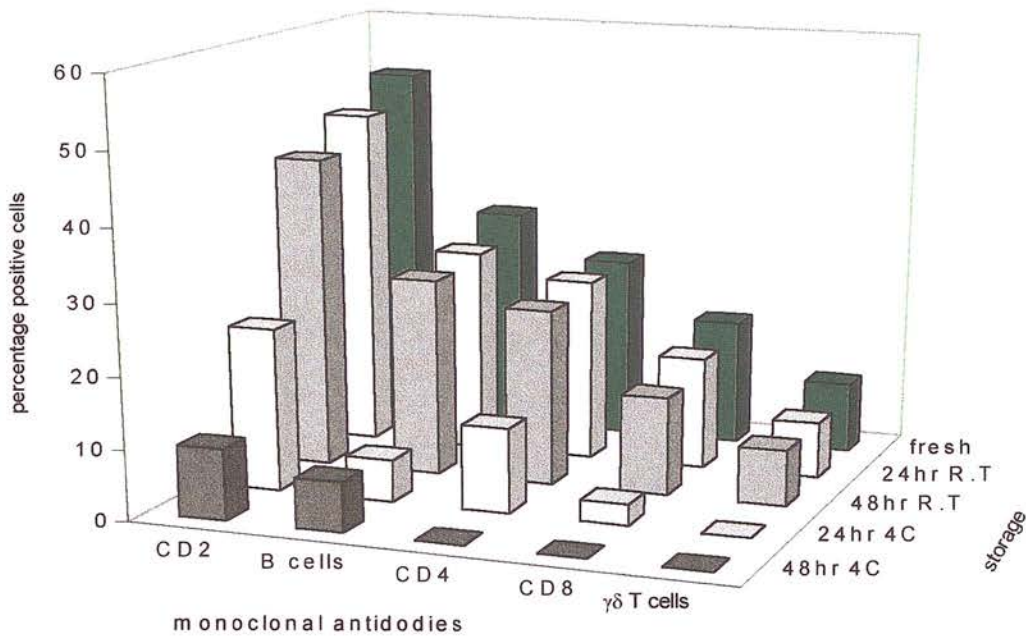


Figure 2.9 PBMC phenotyping after the storage of whole blood samples. The phenotypic profile of the PBMC purified from freshly collected blood was compared with blood after storage for 24 and 48 hours at room temperature (R.T.) and 4°C. The PBMC were immunolabelled with mouse anti-sheep monoclonal antibodies recognising CD2, CD4, CD8, γδ T cells and B cells, and the results were determined by flow cytometry. *Vertical axis:* percentage positive lymphocyte subsets in peripheral blood. *1st horizontal axis:* panel of monoclonal antibodies *2nd horizontal axis:* storage conditions. The results are shown as percentage positive cells/blood.

2.6.1.2 The effect of whole blood storage on PBMC lymphoproliferation

The proliferative response to PHA stimulation (section 2.5.1) was measured for PBMC purified from freshly collected blood samples, and after the heparinised blood had been stored for 24 and 48 hours at room temperature and 4°C. The lymphoproliferative response of the PBMC from fresh blood generated 24306 counts per minute (cpm). After storage of the whole blood for 24 hours at room temperature, the response was reduced by 20%. However, after storage for 48 hours

the response detected was only 45% of the response detected with the fresh blood samples. Whole blood stored at 4°C for 24 and 48 hours resulted in a complete loss of lymphoproliferative response to PHA stimulation (Figure 2.10). These findings conclude therefore that ovine blood could be stored for up to 24 hours at room temperature for lymphoproliferation assays.

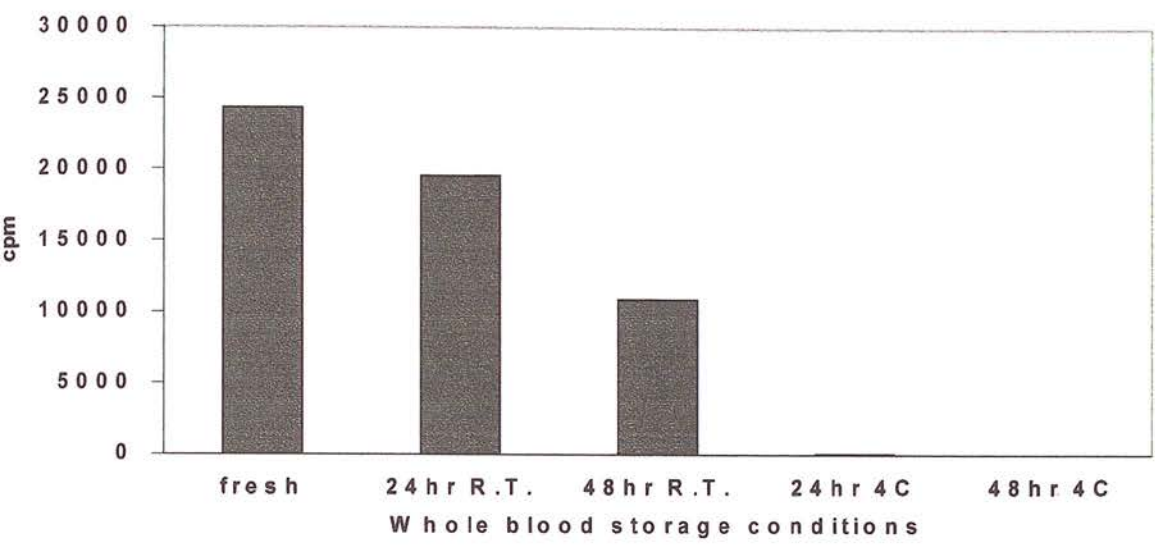


Figure 2.10 Lymphoproliferation after storage of whole blood samples, prior to PBMC purification. The lymphoproliferative response to PHA stimulation was measured in the PBMC purified from freshly collected whole blood and compared with the response generated after storage for 24 and 48 hours at room temperature (R.T.) and 4°C. Results are shown as counts per minute (cpm), determined by ³H-thymidine uptake with unstimulated PBMC (fresh blood 310, after storage for 24 hours: R.T. 320, 48 hours R.T. 178, 24 hours 4°C 56, 48 hours 4°C 42 cpm) subtracted.

2.6.1.3 The effects of storage on immunolabelled, formalin-fixed PBMC.

PBMC were immunolabelled as detailed in section 2.3.1.1 with a panel of mouse anti-sheep monoclonal antibodies recognising CD2, CD4, CD8, $\gamma\delta$ T cells and B cells. The immunolabelled cells were then fixed in 10% formol saline and stored at 4°C. The phenotypic profile was determined by flow cytometry (section 2.3.1.2) at

24, 48, 56, 72 and 96 hours post-labelling, with samples maintained at 4°C between tests.

No detrimental effects on the percentage of positively immunolabelled cell subsets were identified up to 48 hours. A decrease in several subsets, particularly CD8 and $\gamma\delta$ T cells was identified from 56 hours (results not shown). These findings are in accordance with the observations of Lloyd *et al* (1995). As a result of these findings, flow cytometry of samples in all subsequent experiments was performed within 56 hours of immunolabelling.

2.6.1.4 Optimal conditions for venous blood samples

As the blood samples required to be stored at room temperature, and considering variations in ambient temperature, this protocol may not be relevant particularly during seasonal extremes. Moreover, to maintain consistency during monitoring programmes, one method was selected and upheld throughout. It was concluded therefore, that throughout this project all comparative studies would utilise PBMC purified from freshly collected blood samples. For other individual experiments, the PBMC would be purified within 24 hours of blood sample collection.

2.7 Detection of IFN- γ in plasma

The plasma retained from venous blood samples collected during experimental JSRV infection of conventionally housed and SPF lambs (section 2.1.2) was stored at -20°C in 500 μ l aliquots. The level of IFN- γ in the plasma of the JSRV-infected and control lambs was estimated using the sandwich enzyme immunoassay, Bovine Gamma Interferon Test (BOVIGAM™), a bovine IFN- γ detection test which

also recognises ovine IFN- γ (CLS, Melbourne Australia). The tests were performed following the manufacturer's instructions, using the reagents provided. All samples were prepared in duplicate by dispensing 50 μ l of each test plasma, the positive bovine IFN- γ and the negative bovine IFN- γ controls into the 96 well plates. The samples were diluted with 50 μ l plasma diluent buffer and each sample was mixed by pipetting up and down 5 times. The plate(s) were covered and incubated at room temperature for 60 ± 5 minutes. The contents of the wells were shaken out and filled with wash buffer, emptied and re-filled (6 times in total). After the final wash the plates were placed face down on tissue paper and allowed to drain. The conjugate solution was diluted as directed, and 100 μ l was added to each well. The plate(s) were covered and incubated at room temperature for 60 ± 5 minutes and then washed as detailed above. The enzyme substrate solution was prepared by diluting the chromogen in enzyme substrate buffer, and 100 μ l/well was added. The plates were covered with aluminium foil and incubated at room temperature for 30 minutes. After incubation, the enzyme stopping solution was added at 50 μ l/well. The results were determined by optical density at 630nm, within 5 minutes of stopping the reaction. (Dynatech MR5000 microplate reader). The test was validated by determining the mean absorbance for the positive and negative control samples, within the acceptable range of means: the negative bovine IFN- γ control (<0.130), each replicate must not vary >0.040 . The positive bovine IFN- γ control (>0.700), the replicates must not deviate by more than 30% from the mean absorbance. The mean absorbance was calculated for each of the samples and compared to the negative control. Plasma samples with a mean absorbance value in excess of 0.050 of the negative control

mean were compared to the positive control mean absorbance value. All plasma samples within the range of the negative control were considered negative for IFN- γ .

2.8 Carboxyfluorescein diacetate succinimidyl ester (CFSE) proliferation assay

Carboxyfluorescein diacetate succinimidyl ester (CFSE) is a fluorescent dye, which possesses identical spectral characteristics to the fluorochrome, fluorescein. The addition of the reagent pluronic increases the permeability of the cell membrane, allowing the CFSE to be internalised and incorporated into the lymphocytes. The CFSE is stably retained within the cytoplasm of lymphocytes that have been stimulated to undergo mitotic division. Serially, half the fluorescence is inherited by each subsequent generation of cells. The carboxyl groups present in the CFSE compound are cleaved by cytoplasmic esterases, allowing the fluorescent intensity (directly proportional to the CFSE concentration) to be determined for each generation of proliferating cells by flow cytometry. The data generated can be extended further to identify the lymphocyte subsets responding to mitogen stimulation. The PBMC are counter-labelled with a panel of monoclonal antibodies recognising lymphocyte subsets, and the concurrent use of rabbit anti-mouse immunoglobulin/phycoerythrin (Ig/PE) (Sigma, Poole, Dorset) for the secondary antibody facilitates analysis by flow cytometry (Lyons 1999 and references contained therein).

2.8.1 PBMC preparation

PBMC recovered after Lymphoprep™ separation (section 2.2.1(i)) were re-suspended in 10ml ice-cold Hanks balanced salts solution (HBSS) (Life Technologies Ltd., UK) with 10units/ml heparin added. They were centrifuged at

300g for 15 minutes and the supernatant decanted. The PBMC were re-suspended in 10ml ice-cold HBSS and centrifuged again at 300g for 15 minutes. The cells were then re-suspended in 1ml HBSS, counted as detailed in section 2.2.2, and adjusted to a final concentration of 2×10^7 PBMC/ml. The cells were divided into two 15ml conical tubes (labelled CFSE⁺ and CFSE⁻), and the total volume of PBMC in each tube recorded.

2.8.2 Preparation of CFSE

In a waterbath, 1 vial of commercially purchased CFSE and 1 vial of pluronic (both from Molecular Probes, Oregon, USA) were brought to 37°C. Each vial was microcentrifuged for 1 minute and 20µl pluronic added to the CFSE. The sample was mixed thoroughly. A 10µl volume of CFSE/pluronic mix was added to 20ml HBSS, (final concentration 5µM CFSE). Followed by vortexing and filter sterilisation (0.22µm micropore filter).

2.8.3 CFSE labelling

An equal volume of CFSE/HBSS was added to CFSE⁺ samples and an equal volume of HBSS only was added to CFSE⁻ samples. Both samples were incubated at 37°C in 5% CO₂ for 15 minutes. The reaction was stopped by adding an equal volume of ice-cold FCS to both samples. The samples were centrifuged at 180g for 20 minutes at 4°C and the supernatants decanted. The cells were re-suspended in 8ml RPMI-1640 containing 10% FCS and centrifuged at 180g for 10 minutes at 4°C. This was repeated 2 times (3 washes in total). The cells were brought to a final concentration of 10^6 PBMC/ml RPMI-1640 medium containing antibiotics and 10% foetal calf serum.

2.8.4 Lymphoproliferation

The required number of 96 well, round bottomed plates were labelled CFSE⁺ and CFSE⁻, with the mitogen used and the identity of the monoclonal antibody that would be used to counter-label the cells (duplicate wells for each monoclonal antibody). Mitogens (at the optimum concentrations) were added to the PBMC and the samples at 2×10^5 cells/well were dispensed. The plates were incubated at 37°C in 5% CO₂ for 96 hours. After incubation the duplicate wells were pooled into labelled test tubes and 1ml PBS was added. The samples were centrifuged at 300g for 5 minutes. The supernatant was decanted.

2.8.5 Monoclonal antibody counter-staining for lymphocytes subset selection

Monoclonal antibodies specific for CD2, CD4, CD8, $\gamma\delta$ T cells and B cells (at optimum titres Chapter 3, section 3.2) were added to appropriately labelled tubes. The samples were incubated for 30 minutes at 4°C. After incubation 1ml PBS was added to each tube and centrifuged at 300g for 5 minutes. The supernatant was decanted and 100 μ l (1:20 dilution) rabbit anti-mouse Ig/PE was added. The samples were incubated for 30 minutes at 4°C. To each tube 1ml PBS was added and centrifuged at 300g for 5 minutes. The supernatant was decanted and 500 μ l PBS and 100 μ l 10% formol saline added. The samples were stored at 4°C for a maximum of 48 hours.

2.8.6 Phenotyping by flow cytometry

The results were determined and analysed by flow cytometry, using a four colour, twin laser fluorescence activated cell sorter, FACSCalibur™ (Becton Dickinson, New Jersey, USA) with an argon laser set at 488nm. Cell debris was excluded using a forward and side scatter gate. The cells were acquired and the data were generated using Cellquest™ (Becton Dickinson, New Jersey, USA) software,

with a minimum of 2000 events per sample counted. Computer generated scatter profiles and histograms of proliferating PBMC subset generations (fluorescence-1 (FL-1, CFSE concentration)) were obtained to identify the percentage of positive cells (fluorescence-2 (FL-2, PE)) immunolabelled with the monoclonal antibodies.

2.9 Data analysis

2.9.1 Phenotyping (section 2.3).

Percentage positive immunolabelled cells in sheep blood Results expressed as the percentage of immunolabelled cells in peripheral blood were computer generated directly during analysis by flow cytometry. Comparative analysis of the animal groups was determined by calculating the mean percentage of cells labelled positive with each monoclonal antibody tested, \pm standard error of the mean (SEM). A two-tailed student's t-test determined the level of significance between each group.

Proportion of immunolabelled cells per ml blood Where phenotyping data were expressed as the actual number of immunolabelled cells per ml blood, the number of cells detected by flow cytometry, that were positively immunolabelled with each of the monoclonal antibodies tested was calculated as follows:-

$$\frac{\text{numbers of positively labelled cells}}{\text{total number of events}} = \text{proportion of labelled cells in the sample}$$

proportion of labelled cells in the sample \times PBMC/ml blood (cell count, section 2.2)
= number of immunolabelled cells/ml blood.

Group results: the mean number of immunolabelled cells per ml blood for each group was determined, \pm SEM. A two-tailed student's t-test determined the level of significance between each group.

2.9.2 Lymphoproliferation (section 2.5)

The results of the lymphoproliferation assays were expressed as counts per minute (cpm), determined by ^3H -thymidine uptake. For sheep lymphoproliferation assays three replicates of each sample were prepared, and five replicates for the experiments with human blood. In each experiment the mean cpm of the sample replicates, and the control mean cpm were calculated. The results were shown as the mean cpm with the control mean cpm subtracted. Group results: the mean cpm of each group was determined, \pm SEM. A two-tailed student's t-test determined the level of significance between each group.

2.10 Tissue fixation and preservation

Tissue samples (5-6mm thick) collected at necropsy from adult OPA cases and experimentally JSRV-infected lambs (section 2.1) were preserved in a range of fixatives for subsequent immunohistochemistry.

2.10.1 Zinc salts fixative (ZSF)

Zinc salts fixative (ZSF) was initially prepared by adding 0.05% calcium acetate to 0.1M Tris base. The pH was adjusted to 7.4 – 7.6, followed by the addition of 0.5% zinc acetate and 0.5% zinc chloride. The tissues were fixed for 4 – 8 hours and then trimmed to 2 – 3mm thickness, placed in cassettes and post-fixed in fresh ZSF for a further 24 --72 hours. The tissues were then placed in cassettes and immersed in 70% ethanol for 30 minutes.

2.10.2 Alcohol/zinc acetate (AZA)

An equal volume of 70% ethanol was added to ZSF, and the tissues fixed as detailed above.

2.10.3 Aldehyde fixatives

Formaldehyde: Commercial 40% formaldehyde (Becton Dickinson, Rutherford, NJ, USA) was added to phosphate buffered saline (PBS) to final dilutions of 4%, 2% and 1%.

Paraformaldehyde: In a fume cupboard, 24g of paraformaldehyde was dissolved in 550ml of heated PBS, followed by filtration through a Whatman No 1 filter paper. The final volume was adjusted to 600ml with PBS (final dilution of 4%). Paraformaldehyde was stored at 4°C for a maximum of three days.

2.10.4 Cryopreservation

The tissue samples (5 –6 mm) were placed in aluminium foil containers containing OTCT[™] (Thermo Shandon, Pittsburgh PA, USA); more OTC was added to cover the tissues, which were then snap frozen in liquid nitrogen. The tissues were stored at –70°C until required.

2.11 Preparation of fixed tissues for immunohistochemistry

2.11.1 Paraffin wax embedding

Following fixation in ZSF or aldehydes, the tissues were embedded in paraffin wax by an automated process (Hypercentre X processor, Thermo Shandon, Pittsburgh PA, USA). Firstly the tissues were dehydrated by passing through graded alcohols (80% ethanol, 45 min; 95% ethanol, 75 min; 99% ethanol, 3 x 75 min; isopropanol, 2 x 90 min; isopropanol/xylene [1:1 v:v], 90 min; xylene 2 x 90min). The tissues were then embedded in medium temperature [56 – 58°C] paraffin wax, (2 x 105 min).

2.11.2 Dewaxing ZSF and aldehyde-fixed tissues

Tissue sections 5µm thick were cut on a microtome, and placed on to Superfrost Plus™ treated glass sides (Menzel-Glaser, Germany) and dried at 37°C. The tissues were dewaxed and rehydrated by placing the slides in xylene, (2 x 5 min), 100% ethanol, 95% ethanol, 75% ethanol (5 min each). This procedure was performed immediately prior to immunolabelling or antigen retrieval.

2.11.3 Antigen retrieval of aldehyde-fixed tissues

- (i) Microwave oven: Antigen retrieval by boiling the tissues in a microwave oven was attempted using a method adapted from previously reported protocols (Shi *et al* 1981, Gown *et al* 1993, Sibony *et al* 1995, Pileri *et al* 1997). The tissues were dewaxed and rinsed in 0.85% NaCl for 5 min. The slides were placed in a plastic, microwavable rack and immersed in sodium citrate buffer (12ml 0.1M citric acid and 48ml 0.1M tri-sodium citrate in 600ml distilled water, pH 6.0) in a glass container. The slides were heated, on full power, in a 650 – 700W microwave oven for 7 minutes (checking fluid level after 4 minutes). After heating the slides were allowed to cool in the buffer for 20 minutes and then rinsed in PBS, 2 x 5 min. Several attempts at antigen retrieval by the microwave method were performed with the following alterations to the protocol. After rehydration, the NaCl step was omitted and the slides immersed in PBS. The microwave time was increased to 10 and 15 mins, adding more buffer every 5 minutes. Citrate buffer was replaced with disodium salt dihydrate buffer (10mM EDTA, PH 8.0) (Leong *et al* 1998) as the retrieval solution. Combinations of the above changes to the protocol were tested.

- (ii) Pressure-cooking: Antigen retrieval by heating the tissues in a pressure cooker was attempted using a method adapted from previously reported protocols (Norton *et al* 1994, Miller *et al* 1995). The tissues were dewaxed as detailed above, and placed in Tris-buffered saline (TBS). Two litres of citrate buffer (as previously described) were brought to boiling point in a pressure cooker (Prestige Group, UK), without sealing the lid. The slides were placed in metal racks (evenly spaced) and lowered into the buffer. The pressure cooker lid was sealed and weighted. When full pressure was reached, heating was timed for 5 minutes. The pressure cooker was depressurised in cold running water. The slides were removed and placed under running tap water and then transferred to TBS.

2.12 Cryostat sections

The cutting chamber in the cryostat (Cryostat 2800 Frigocut-E) was adjusted to -15°C , the recommended temperature for lung and lymph nodes. All instruments, brushes, needles, forceps, etc., were placed and retained in the cutting chamber. The tissues were removed from -70°C storage and brought to -15°C . The specimen stage (holding device for the tissue block) was kept at room temperature. A small amount of cryocompound was applied to the specimen stage to attach the tissue block. The specimen stage and attached tissue were then placed in the cutting chamber to ensure good adhesion between the specimen and the specimen stage. Tissue sections of 8 – 10 μm were cut by adjusting the position of the blade to the required thickness, and aligning the tissue and anti-roll guide. Depressing the foot pedal moved the tissue vertically; the cut section was then placed on the Superfrost PlusTM treated glass

slide. The sections were air dried and fixed in acetone for 15 min, allowed to air dry again and stored at -4°C until required. Cryopreserved sections were rehydrated by passing the slides through graded alcohols as described for aldehyde fixed tissues. This procedure was performed immediately prior to immunolabelling.

2.13 Immunohistochemistry

2.13.1 Immunolabelling tissue sections

The tissues were labelled with mouse anti-sheep monoclonal antibodies and the positively immunolabelled cells were detected microscopically by the EnVision PlusTM detection system (DAKO, Ely, Cambridge, UK). The entire immunohistolabelling procedure was performed using a vertical staining system, SequenzaTM slide rack and COVERPLATE system (Shandon, Runcorn, UK). After rehydration, dewaxing (and antigen retrieval, where appropriate), the fixed or cryopreserved tissue sections were immersed in TBS buffer for 5 mins. The area of slide around each section was dried using a tissue. The slides were placed in a COVERPLATETM immunostaining chamber and inserted into the SequenzaTM slide rack. The reservoir was immediately filled with TBS and allowed to drain through. To each reservoir 100 μl peroxidase block (0.03% hydrogen peroxide in methanol) were added and incubated at room temperature for 5 minutes. The slides were then washed twice by filling the reservoirs with TBS and allowing to fully drain. To block non-specific antigens 100 μl of 25% normal goat serum in TBS were added and incubated at room temperature for 30 minutes. The slides were then washed twice with TBS and 100 μl of mouse anti-sheep monoclonal antibodies were added at the optimum (or required) dilutions. The racks were covered and incubated at 4°C

overnight. The slides were washed twice in TBS and 100µl of secondary antibody (peroxidase-labelled polymer conjugated to goat anti-mouse immunoglobulins, supplied in the EnVision Plus™ detection kit were added to each reservoir and incubated at room temperature for 30 mins. The slides were washed twice with TBS buffer. The substrate chromogen, 3,3'-diaminobenzidine (DAB), supplied in the detection kit, was added at 100µl per slide and incubated at room temperature for 7.5 mins. The slides were washed twice in distilled water and then removed from the COVERPLATES and immersed in haematoxylin at room temperature for 1 min, rinsed in distilled water and immersed in Scotts tap water solution (STWS) at room temperature for 1 min and rinsed again in distilled water. The tissues were dehydrated by passing through graded alcohols (75% ethanol; 95% ethanol; 100% ethanol; xylene x 2, all for 5 mins at room temperature). Coverslips were applied to the slides using DPX mountant.

Chapter 3

Systemic immune responses

**following infection with jaagsiekte sheep retrovirus
and in the terminal stages of ovine pulmonary adenocarcinoma.**

Introduction

The immunological responses during JSRV infection are poorly understood. No JSRV-specific circulating antibodies have been detected in adult clinical cases of OPA (Sharp and Herring 1983, Ortin *et al* 1998) or during experimentally induced JSRV-infection (van der Molen personal communication). Furthermore, no systemic cell-mediated responses have been identified, thus far. There are no field tests currently available to diagnose JSRV infection prior to the onset of clinical disease: consequently monitoring the immune responses of adult JSRV-infected sheep during tumorigenesis has been impeded. However, clinical and pathological OPA can be experimentally induced in neonatal lambs by intratracheal inoculation with virus purified from tumour (Martin *et al* 1976), lung fluid (Sharp *et al* 1983) or a JSRV infectious molecular clone (Palmarini *et al* 1999, DeMartini *et al* 2001), giving the opportunity to follow any immunomodulations temporally.

In this investigation, it was proposed to evaluate the systemic immune responses at all stages of disease, from experimentally induced JSRV infection and tumorigenesis in lambs to the terminal stages of OPA in adult sheep. For all animals, experimental lambs and adult sheep alike, the peripheral blood leukocytes were characterised to identify phenotypic frequency alterations, which may indicate an immunological response during JSRV infection. *In vitro* lymphoproliferation to mitogen stimulation was performed to identify any alteration in cellular function during JSRV infection. Evidence of any changes in the concentration of IFN- γ in the blood of experimental lambs was determined also. The experimental procedures are summarised in Figure 3.1.

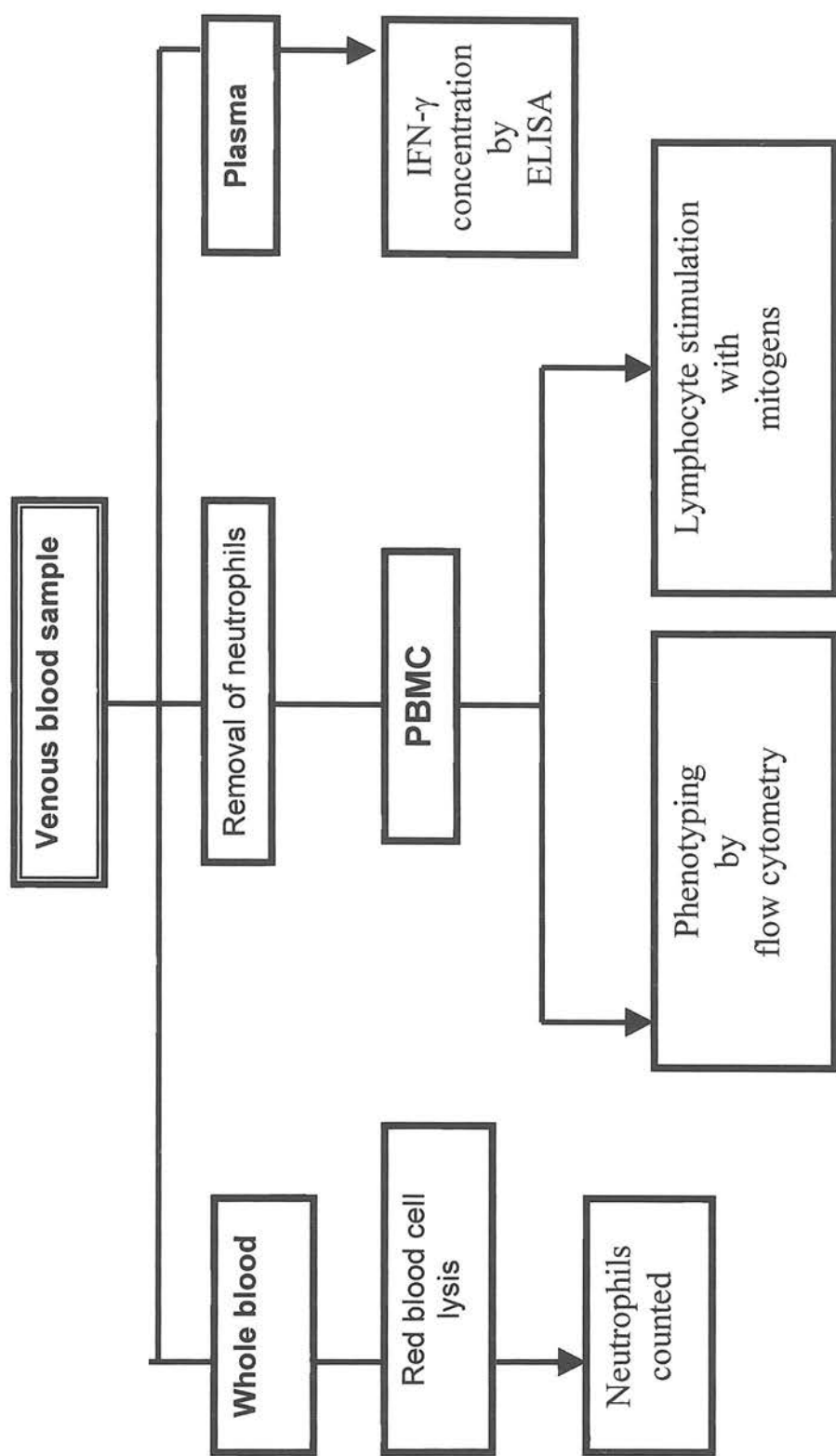


Figure 3.1 Experimental procedures to measure phenotypic and functional changes in the peripheral blood of sheep with JSRV infection

Adult JSRV-infected sheep, in the terminal stages of OPA A CD4⁺lymphocytopaenia and neutrophilia have been demonstrated previously in the peripheral blood in such sheep (Rosadio and Sharp 1992, Holland *et al* 1999). It also has been established that a JSRV-disseminated infection occurs with viral RNA and proviral DNA found in cells of the immune system (Holland *et al* 1999). In the study reported here (section 3.1) the immunological status in the terminal phase of OPA was measured by characterising the phenotypic profile and responses to mitogen stimulation in a single blood sample collected from clinical cases prior to humane killing, and compared to blood collected from adult control animals without OPA.

Experimental JSRV infection of neonatal lambs To monitor systemic immune responses during the pre-clinical stages of disease two experiments were performed with: (i) conventionally housed lambs and (ii) specific pathogen free (SPF) lambs. Experimental infection was induced in the neonatal lambs by intratracheal inoculation with JSRV purified from lung fluid. The remaining age-matched lambs in each experiment acted as controls throughout. Serial blood samples were collected until the onset of clinical disease. When symptoms of OPA were apparent, the animals were killed and necropsy performed. Macroscopic and histological examination of tumour and lung tissue confirmed the disease status of each animal (section 3.2).

Phenotyping analysis To determine the peripheral blood leukocyte (PBL) phenotypic characteristics of adult JSRV infected sheep, in the terminal stages of OPA (section 3.1.1) and to monitor any phenotypic frequency alterations in the PBL of experimentally infected lambs (section 3.2.3) the cells were phenotyped using a panel of monoclonal antibodies recognising CD2, CD4, CD8, $\gamma\delta$ T cells, B cells and

MHC class II antigens and the results determined by flow cytometry. Neutrophils were counted also.

Lymphoproliferation: To identify any changes in cell-mediated responses in the adult OPA cases and during experimental JSRV infection, *in vitro* lymphoproliferation following stimulation by various mitogens was measured. The response to phytohaemagglutinin (PHA) and concanavalin A (ConA) stimulation was measured in adult sheep with OPA and compared with the response detected in adult controls (section 3.1.2). The lymphoproliferative responses to PHA, ConA and pokeweed mitogen (PWM) stimulation were monitored in the blood samples collected from the lambs experimentally infected with JSRV and control lambs (section 3.2.4).

Identification of PBMC subsets responding to PWM and ConA stimulation The aim of this study was to determine which lymphocyte subsets were induced to undergo mitotic division by stimulation with PWM and ConA (section 3.3). The lymphocyte subsets proliferating in response to PWM and ConA stimulation were identified by flow cytometry using internalised carboxyfluorescein diacetate succinimidyl ester (CFSE) and monoclonal antibody selection.

Requirement for antigen presentation in mitogenicity: The objective of this study was to determine if the mitogenicity of PHA, ConA and PWM was dependent on antigen presentation (section 3.4). The lymphoproliferative response to the mitogens was measured and compared in the entire-PBMC population, in PBMC depleted of adherent cells and in reconstituted-PBMC (adherent cells replaced), all derived from the same blood samples.

IFN- γ levels detected in the blood of experimentally JSRV-infected lambs: It was hypothesised that changes in the concentration of IFN- γ detected in the blood of JSRV-infected lambs could indicate activation of an immunological response or that cytokine alterations were induced as a result of the infection. The level of IFN- γ in the blood samples collected during the experimental JSRV infection of conventionally housed and SPF lambs was measured and compared with the level in the uninfected animals (section 3.5).

3.1 Adult sheep in the terminal stages of OPA

The systemic immune system of adult sheep in the terminal stages of OPA was evaluated by comparing the phenotypic profile and cellular responses in the peripheral blood, with blood samples collected from uninfected control animals from the Moredun Research Institute. Details of all the sheep involved in this study are described in Chapter 2, section 2.1.1.

3.1.1 The phenotypic profile of peripheral blood leukocytes in adult sheep, in the terminal stages of OPA

3.1.1.1 Methodology

The phenotypic profile of the peripheral blood leukocytes was characterized from venous blood samples collected (Chapter 2, section 2.1.4) from twelve adult OPA case in the terminal stages of disease and ten adult control sheep, age-matched where this information was available. Aliquots of 100 μ l whole blood were retained for the neutrophil counts as detailed in Chapter 2, section 2.4. The PBMC were purified (Chapter 2, section 2.2.1) and adjusted to a final concentration of 10^6 PBMC/ml. The PBMC were phenotyped using a panel of mouse anti-sheep

monoclonal antibodies specific for CD2, CD4, CD8, $\gamma\delta$ T cells, B cells and MHC class II antigens and the results were determined by flow cytometry (Chapter 2, section 2.3). The data were analysed and group results compiled as detailed in Chapter 2, section 2.9.

3.1.1.2 Altered immune cell populations are demonstrated in adult sheep with terminal OPA

The results for each of the cell subsets are shown in Figure 3.2. The percentage of positively immunolabelled CD2 T cells ($42\% \pm 3$) in the lymphocyte region of the PBMC from the OPA group was significantly decreased ($p = 0.02$) in comparison with the percentage ($52\% \pm 3$) detected in the control group. The reduced T cell population was further characterised by a statistically significant ($p=0.004$) reduction in the percentage of positively immunolabelled CD4 T cells in the blood of the OPA sheep ($16\% \pm 2$) compared with the control animals ($24\% \pm 2$). The percentage of neutrophils present in the blood of the OPA sheep ($52\% \pm 6$) was significantly higher ($p=0.05$) compared with percentage detected in the control animals ($33\% \pm 6$). No statistically significant difference in the percentage of immunolabelled cells was identified for CD8 T cells, $\gamma\delta$ T cells, B cells or MHC class II antigens, between the two animal groups.

3.1.2 Lymphoproliferation in response to mitogen stimulation in adult sheep in the terminal stages of OPA

3.1.2.1 Methodology

The PBMC were purified (Chapter 2, section 2.2) from blood samples collected from ten adult sheep in the terminal stages of OPA and ten adult control animals. Lymphoproliferation following stimulation with PHA and ConA was measured as detailed in Chapter 2, section 2.5 and the level of response to each

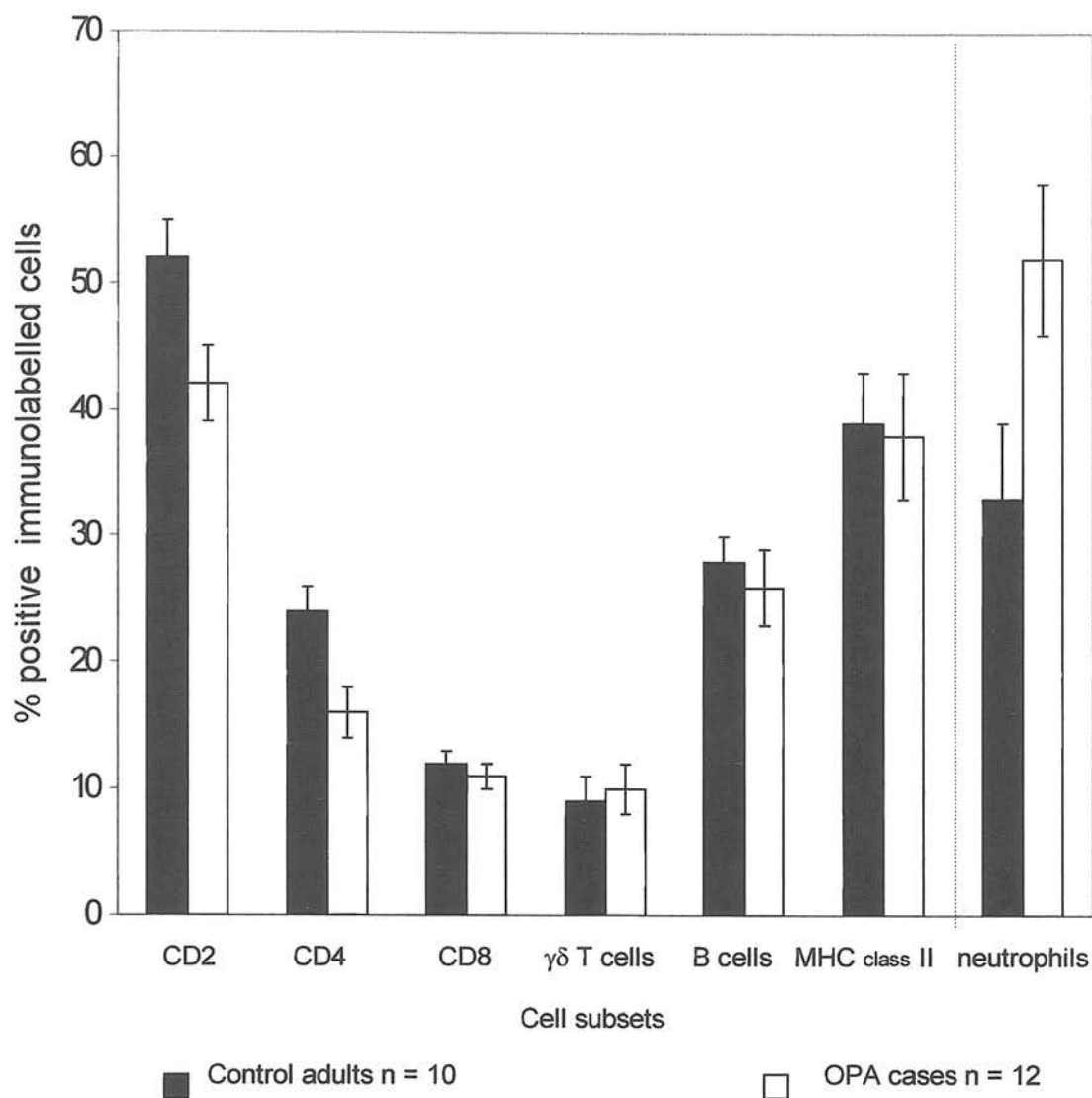


Figure 3.2 The phenotypic profile of the peripheral blood leukocytes from adult sheep with OPA and control sheep. The PBMC were purified from blood samples collected from 12 adult sheep in the terminal stages of OPA and 10 control adult sheep. The PBMC were phenotyped with a panel of mouse anti-sheep monoclonal antibodies specific for CD2, CD4, CD8, $\gamma\delta$ T cells, B cells and MHC class II antigens. The results were determined by flow cytometry. Results for CD2, CD4, CD8, $\gamma\delta$ T cells and B cells are shown as the percentage positively immunolabelled cells/lymphocyte region of the PBMC \pm SEM. Cells in the PBMC expressing MHC class II antigens are shown as the percentage positively immunolabelled cells/PBMC, \pm SEM. The percentage of neutrophils present in the blood was also recorded, \pm SEM.

mitogen between the two animal groups was compared. The data were analysed and group results compiled as described in Chapter 2, section 2.9.

3.1.2.2 The cellular response to ConA stimulation is altered in OPA adult sheep

No significant difference in the level of response to PHA stimulation was detected between the two groups of animals. However, a significantly ($p = 0.03$) reduced response to ConA stimulation was detected in the PBMC purified from the blood of the OPA cases ($10955 \text{ cpm} \pm 2532$) compared with the response detected in the PBMC from the control adult sheep ($19000 \text{ cpm} \pm 2426$) (Figure 3.3).

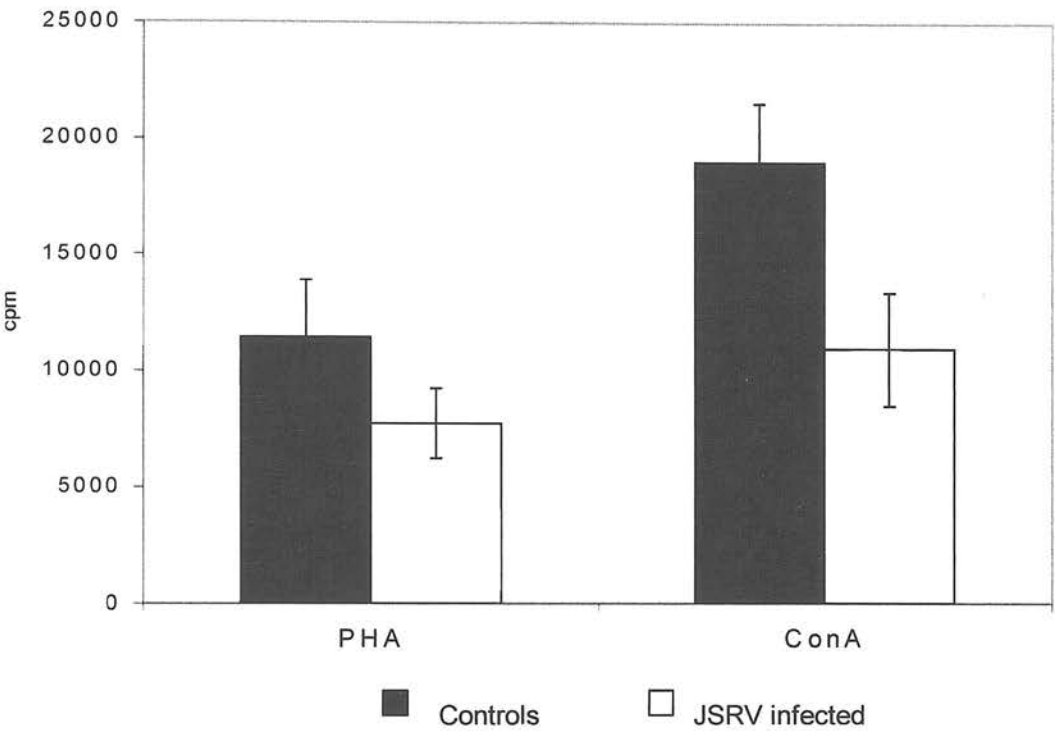


Figure 3.3 Lymphoproliferation to PHA and ConA stimulation in adult sheep with OPA and control sheep. The PBMC purified from the blood of 10 adult OPA cases and 10 control adult sheep were stimulated with PHA and ConA. The results are shown as counts per minute (cpm) \pm SEM, determined by ^3H -thymidine uptake with the unstimulated PBMC: control sheep 326 cpm and OPA cases 304 cpm subtracted. No significant difference in the response to PHA stimulation was detected between the two animal groups. The response to ConA stimulation was statistically significantly reduced ($p=0.03$) with the adult sheep with OPA in comparison with the control animals.

3.2 Experimental JSRV-infection of neonatal lambs

Experimental JSRV infection was induced in six conventionally housed and six SPF neonatal lambs by intratracheal inoculation with JSRV purified from lung fluid (Chapter 2, section 2.1.3). The remaining four lambs in each experiment acted as controls throughout. The two groups of lambs were housed separately. Venous blood samples were collected from the lambs (Chapter 2, section 2.1.4) at weekly intervals until they were ten weeks old and then every two weeks until the onset of clinical disease. When symptoms of OPA were apparent, the animals were killed and necropsy performed. Macroscopic and histological examination of tumour and lung tissue confirmed the disease status of each animal (Chapter 2, section 2.1.5). Details of the JSRV experimental infections are summarized in Figure 3.4.

3.2.1 Conventionally housed lambs

Ten lambs were housed with their mothers under conventional conditions as described in Chapter 2, section 2.1.2 (i). Unfortunately, before weaning when the lambs were five weeks old, two of the dams (mothers of control lambs) developed mastitis, which potentially could have affected their lambs' immunological responses. A coccidia infection was detected in several lambs at approximately two months of age and, although remaining sub-clinical, an immunological response to this concurrent infection could not be disregarded.

Five out of the six conventionally housed lambs experimentally infected with JSRV (Chapter 2, section 2.1.3) developed histopathologically confirmed OPA. Clinical symptoms were diagnosed and OPA confirmed in three lambs at twelve weeks, one lamb at sixteen weeks, and one lamb at twenty weeks old: One JSRV-inoculated lamb with respiratory symptoms, was killed at eighteen weeks of age but

OPA was not confirmed. Two of the control lambs were killed at sixteen weeks and the remaining two controls were killed at twenty weeks of age. Details of the experimental infection and age of lambs, diagnosed with clinical signs of OPA are summarised in Figure 3.4.

3.2.2 Specific pathogen free (SPF) lambs

To minimise interference from possible intercurrent infections and confirm the results obtained with the conventionally housed animals, the experiment was repeated with ten lambs born and housed in SPF conditions as described in Chapter 2, section 2.1.2 (ii). Three out of the six SPF housed lambs, experimentally infected with JSRV (Chapter 2, section 2.1.3), developed histopathologically confirmed OPA. One JSRV-infected lamb died at eleven days old (cause unknown). OPA was confirmed in one lamb at nine weeks, one lamb at eighteen weeks and one lamb at twenty weeks of age. One lamb was killed at fourteen weeks but OPA was not diagnosed. OPA was not confirmed for the remaining JSRV-inoculated lamb, killed at twenty weeks of age. Two control lambs were killed at sixteen weeks and the remaining two control lambs were killed at twenty weeks. Details of the experimental infection and ages of lambs, diagnosed with clinical signs of OPA are summarised in Figure 3.4.

3.2.2.1 Blood clots were detected in the samples from SPF lambs.

Regrettably during this experiment an unexplained problem occurred with the blood samples collected from the SPF lambs. At two weeks of age clots were detected in several of the blood samples, which meant that the functional assays of the PBMC were not carried out on a regular basis. Initially not all lambs were affected, but by five weeks of age clots were evident in all the blood samples

collected from the JSRV-infected and control lambs. Several investigations were performed to identify the cause of this problem.

3.2.2.2 Investigations performed to identify the cause of the clots in the blood of SPF lambs

The blood samples were histologically analysed, and fibrin clots were revealed, containing peripheral blood leukocytes. Biochemical analysis did not indicate any abnormality in the composition of the blood. The preservative-free heparin used in sample collection was tested with blood from adult sheep and conventionally housed age-matched lambs. No clotting was evident in any blood sample examined. Notwithstanding, a new supply of heparin was used but the problem persisted. The technique employed in bleeding the animals was examined but no cause could be identified. The diet of the SPF lambs (Chapter 2, section 2.1.2 (ii)) was reviewed (the SPF lambs were not fed sheep milk, but were given UHT cow milk, with the composition of lactose and lipids differing from ewe milk) and although no specific cause for this phenomenon was confirmed, the problem ceased when the lambs were completely weaned at six weeks old and fed only Lamb Nuts, possibly indicating a dietary cause.

3.2.3 The phenotypic profile of peripheral blood leukocytes, monitored during experimental JSRV-infection

3.2.3.1 Methodology

To monitor any phenotypic frequency alterations in the peripheral blood leukocytes (PBL) of experimentally infected lambs, from inoculation to the onset of clinical disease, venous blood samples were collected as described in Chapter 2, section 2.1.4. The numbers of animals at each time point is shown in Table 3.1.

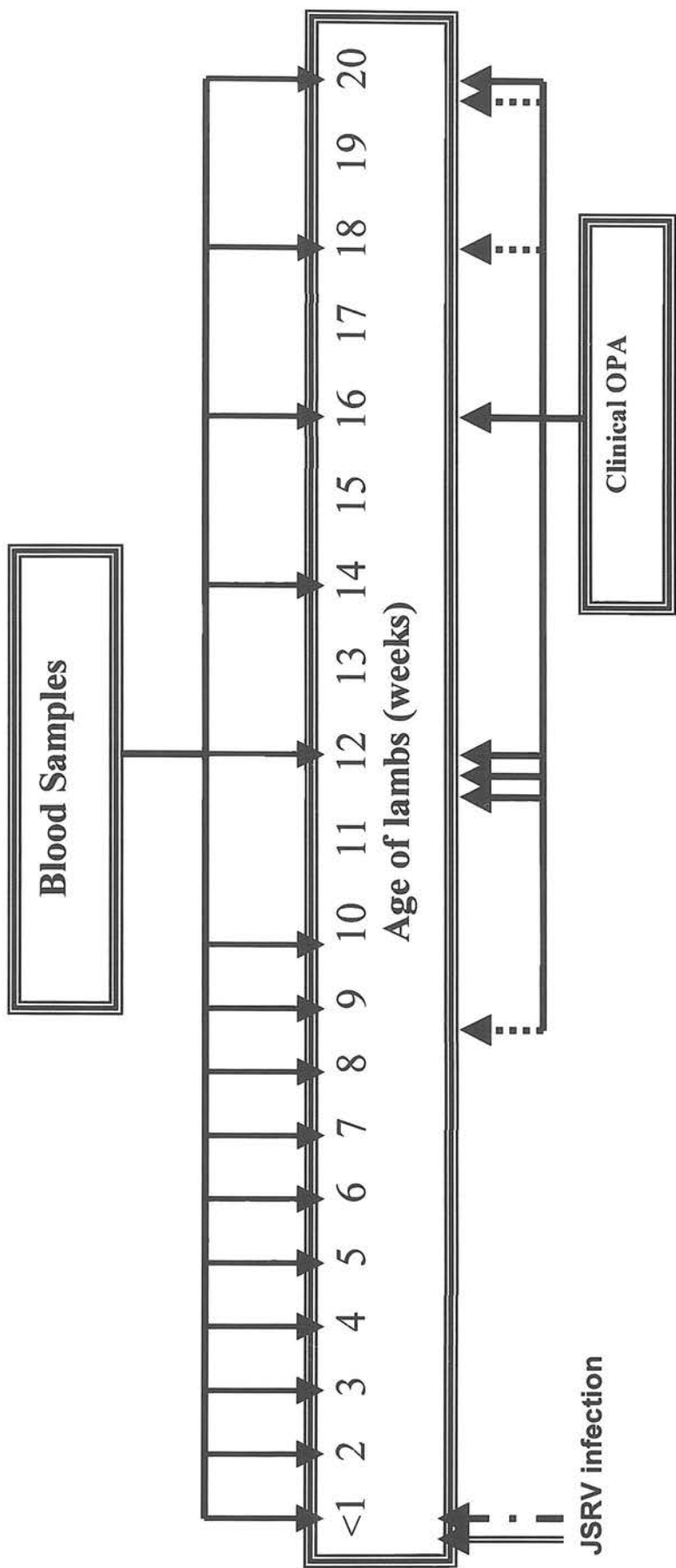


Figure 3.4 Summary of experimental design. Blood samples taken weekly until the lambs were 10 weeks old and every two weeks until 20 weeks of age. The lambs were inoculated with JSRV when they were less than 1 week old. Diagnosis of clinical symptoms: ► conventionally housed lambs and► SPF lambs.

Aliquots of 100µl whole blood were retained for neutrophil counts as detailed in Chapter 2, section 2.4. The PBMC were purified as detailed in Chapter 2, section 2.2.1 and adjusted to a final concentration of 10⁶ PBMC/ml (Chapter 2, section 2.2.2). The PBMC subset frequencies were monitored in both the conventionally housed and SPF lambs by phenotyping with a panel of mouse anti-sheep monoclonal antibodies specific for CD2, CD4, γδ T cells and B cells (Chapter 2, section 2.3.1.1) and the results determined by flow cytometry (Chapter 2, section 2.3.1.2). The data were analysed and group results compiled as described in Chapter 2, section 2.9. The PBMC purified from the blood of the conventionally housed lambs also were immunolabelled with monoclonal antibodies specific for CD8 T cells and MHC class II antigens also. Unfortunately as a result of the problems encountered in the blood of the SPF lambs (section 3.2.2) immunolabelling for CD8 and MHC class II antigens was not performed at all the time points with these animals.

| Sample Numbers | | | | | | | | | | | | | | | |
|-------------------------------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|
| Time points | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 12 | 14 | 16 | 18 | 20 |
| Conventionally housed controls | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 2 | 2 |
| Conventionally housed JSRV infected | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 3 | 3 | 2 | 1 |
| SPF controls | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 2 | 2 |
| SPF JSRV infected | 6 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 4 | 3 | 3 | 2 | 2 |

Table 3.1 Number of animals at each time point during experimental JSRV infection.

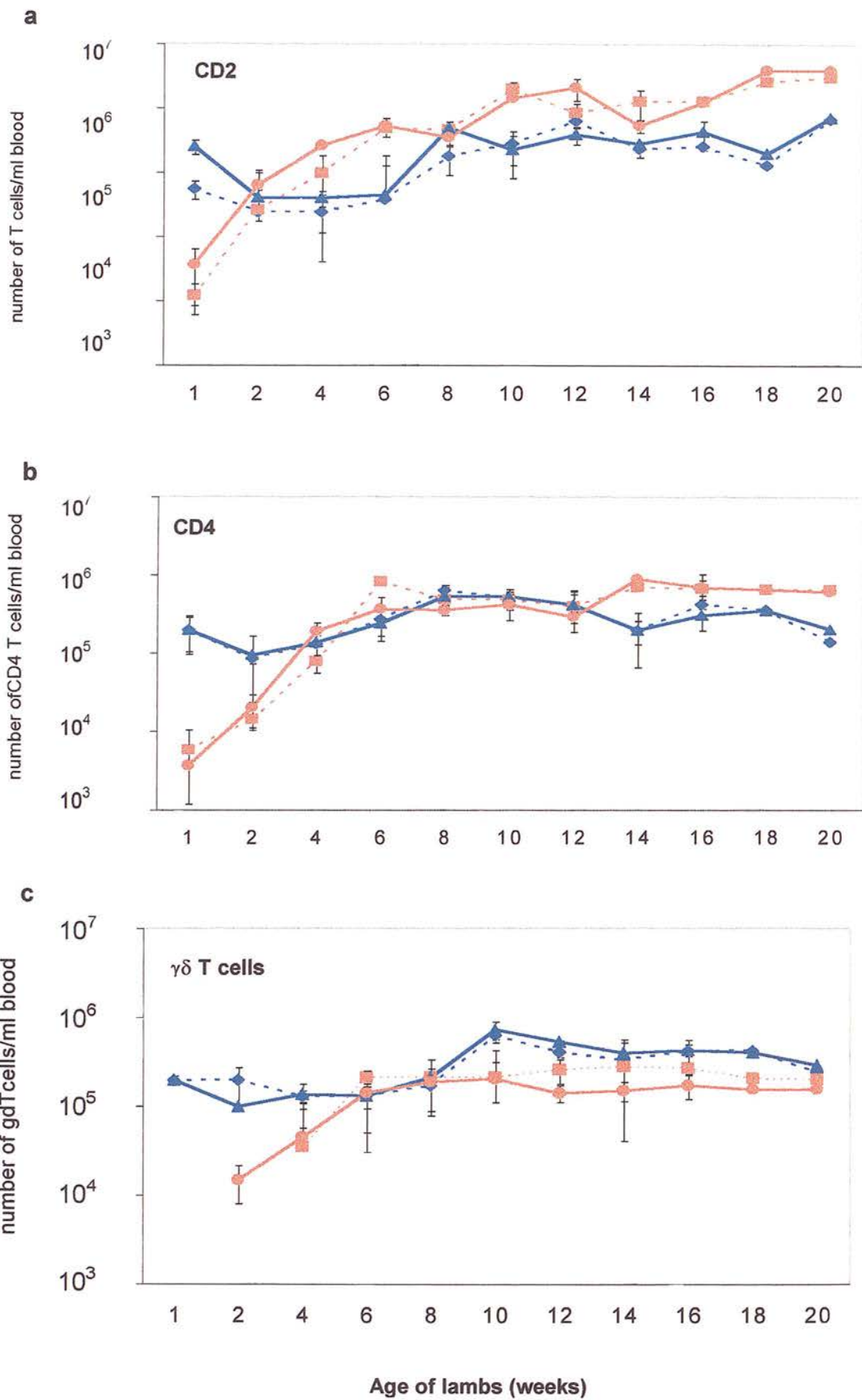
3.2.3.2 No phenotypic alterations in peripheral blood subsets were detected in JSRV-infected lambs

No statistically significant phenotypic alterations were detected in any of the immunolabelled cell populations from the JSRV-infected lambs in comparison with their respective controls, at any time point during the experiments. The phenotypic profiles of CD2, CD4, $\gamma\delta$ T cells and B cells, from conventionally housed and SPF lambs are shown in Figures 3.5a-d, respectively.

Lymphocytopaenia and neutrophilia were not demonstrated in JSRV- infected lambs

No statistically significant differences in the number of positively immunolabelled CD4 T cells/ml blood were detected in the experimentally JSRV-infected compared with the controls lambs, at any time point during the experiments (Figure 3.5b). In addition no alteration in the percentage of neutrophils in the blood was detected in the JSRV-infected lambs, in comparison with the control lambs (Figure 3.5e). Consequently during experimental JSRV infection there was no evidence of the CD4 lymphocytopaenia or neutrophilia previously demonstrated in the peripheral blood of adult OPA cases (Rosadio and Sharp 1992, Holland *et al* 1999) and shown in section 3.1.1.3 of this study.

The phenotypic profiles of CD8 T cells and MHC class II antigens detected in the conventionally housed lambs are shown in Figure 3.6 a and b. No significant differences in the phenotypic profiles were observed between the control and infected lambs, at any time point during JSRV infection. These trends were confirmed, at the time points available, with immunolabelled PBMC from the SPF lambs (results not shown).



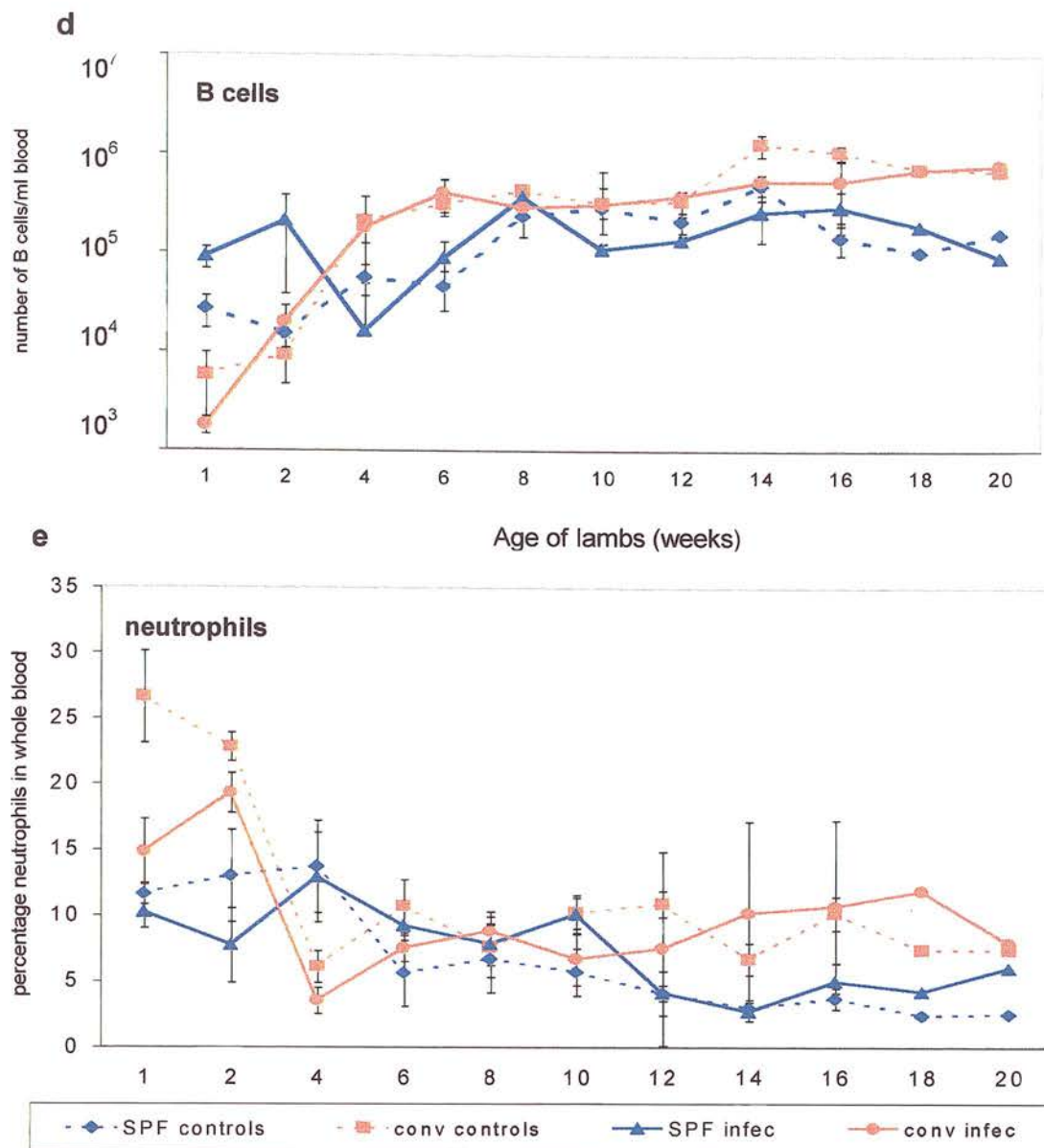


Figure 3.5 Leukocyte subset frequencies in the peripheral blood during experimental JSRV infection. The PBMC were purified from the blood of lambs during experimental JSRV infection: (■) conventionally housed control lambs: 1-16 weeks n=4, 18-20 weeks n=2. (●) conventionally housed JSRV-infected lambs: 1-12 weeks n=6, 14-16 weeks n=3, 18 weeks n=2, 20 weeks n=1. (◆) specific pathogen free control lambs: 1-16 weeks n=4, 18-20 weeks n=2. (▲) specific pathogen free JSRV-infected lambs: 1 week n=6, 2-9 weeks n=5, 10-12 weeks n=4, 14-16 weeks n=3, 18-20 weeks n=2. The PBMC were phenotyped with monoclonal antibodies specific for CD2, CD4, $\gamma\delta$ T cells and B cells, with the results determined by flow cytometry. The results are shown in Figure 3.5 a-d, respectively as the number of positively immunolabelled cells/ml blood. Neutrophils were also counted (Figure 3.5e), with results shown as percentage neutrophils/blood. Each time point represents the group mean \pm SEM.

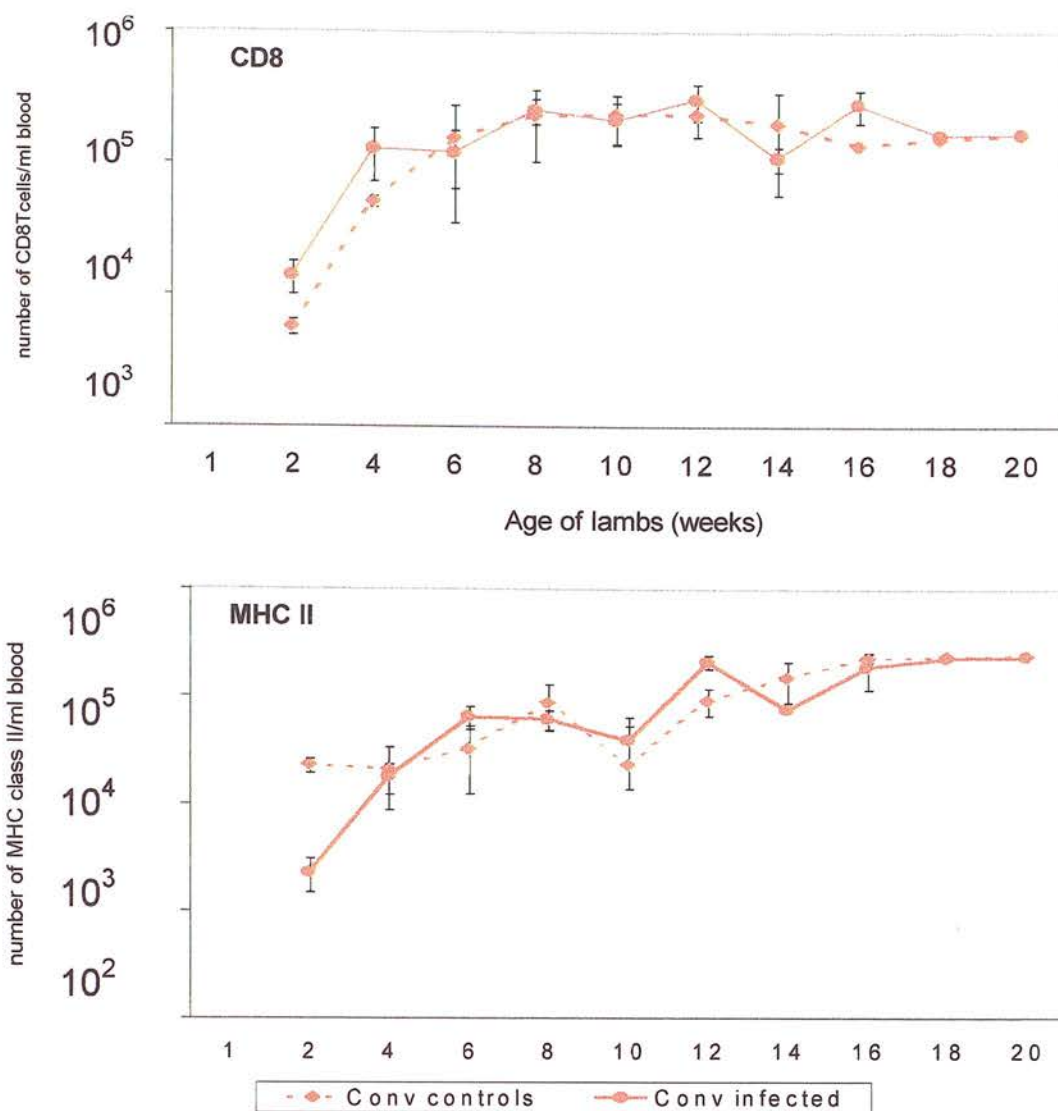


Figure 3.6 CD8 T cells and MHC class II antigen frequencies in the peripheral blood during experimental JSRV infection of conventionally housed lambs. The PBMC were purified from the blood of (■) conventionally housed control lambs: 1-16 weeks n=4, 18-20 weeks n=2. (●) conventionally housed JSRV-infected lambs: 1-12 weeks n=6, 14-16 weeks n=3, 18 weeks n=2, 20 weeks n=1. The PBMC were phenotyped with mouse anti-sheep monoclonal antibodies specific for CD8 T cells and MHC class II antigens, with the results determined by flow cytometry. The results are shown in Figure 3.6 a and b, respectively, as the number of positively immunolabelled cells/ml blood. Each time point represents the group mean \pm SEM.

3.2.4 Lymphoproliferation in response to mitogen stimulation during experimental JSRV-infection.

3.2.4.1 Methodology

During experimental JSRV infection, the PBMC were purified (Chapter 2, section 2.2.1) from the blood of the conventionally housed and SPF lambs. Lymphoproliferative responses to PHA, ConA and pokeweed mitogen (PWM) stimulation were measured (Chapter 2, section 2.5). For each JSRV-infected group the level of response to mitogen stimulation was compared with the response detected in the corresponding control lambs. The data were analysed and group results compiled as described in Chapter 2, section 2.9.

3.2.4.2 The cellular response to ConA stimulation is altered in experimentally JSRV-infected lambs

The lymphoproliferative response to mitogens in the conventionally housed lambs (six JSRV-infected and four control lambs) was measured at four, eight and twelve weeks. At sixteen weeks the response of three JSRV-infected and four controls lambs, and at twenty weeks the response of the remaining JSRV-infected and two control lambs was measured. The results are shown in Figure 3.7. No difference in the lymphoproliferative response to PHA (Figure 3.7a) or PWM (Figure 3.7b) stimulation was detected between the JSRV-infected and the control lambs. In contrast, the infected lambs were less responsive to ConA stimulation than the control animals (Figure 3.7c). This observation was first detected prior to the onset of clinical disease when the lambs were eight weeks of age. The mean lymphoproliferative response detected in the six JSRV-infected lambs was only 34% of the mean response detected in the four control animals. The reduced response to ConA stimulation was evident at all other time points, at twelve weeks the response

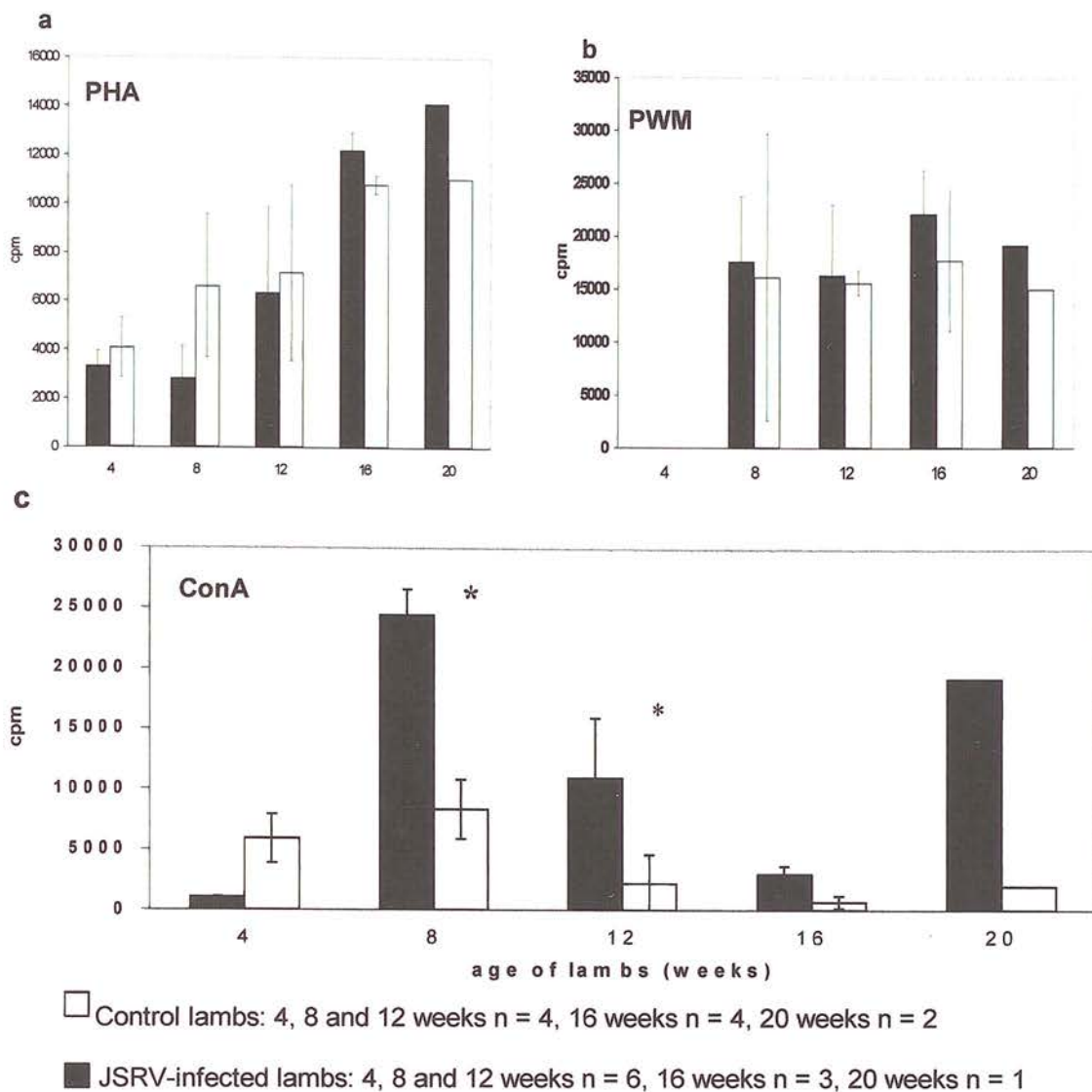


Figure 3.7. Lymphoproliferative response to PHA, PWM and ConA stimulation during experimental JSRV-induced OPA in conventionally housed lambs. PBMC purified from the blood of conventionally housed lambs were stimulated with PHA, PWM and ConA. The results are shown at 4, 8, 12, 16 and 20 weeks. The results are shown as counts per minute (cpm), \pm SEM determined by ^3H -thymidine uptake with the unstimulated PBMC cpm subtracted (4 weeks 171 : 192, 8 weeks 182 : 192, 16 weeks 228 : 243 and 20 weeks 340 : 175 cpm) control lambs and JSRV-infected lambs respectively. The lymphoproliferative response to PHA stimulation is shown in Figure 3.7a, to PWM in Figure 3.7b and to ConA in Figure 3.7c. No significant difference to PHA or PWM was detected between the JSRV-infected and control lambs, at any time point. In contrast statistically significantly (* $p=0.002 - 0.008$) reduced responses to ConA stimulation were detected in the JSRV-infected lambs, compared with the controls. At 20 weeks only one JSRV-infected and two control lambs remained giving cpm of 2207 and mean cpm 19231, respectively.

of the JSRV infected lambs was only 20% of the response identified in the control animals, at sixteen weeks the response of the JSRV-infected lambs was 23%, in comparison with the control lambs. At the end of the experiment at twenty weeks the response detected in the remaining JSRV-infected lamb was 10% of the mean response detected in the two control animals.

The lymphoproliferative responses detected in the SPF lambs confirmed these findings. The results (when available) are shown in Figure 3.8. When the lambs were 4 weeks old the mean lymphoproliferative response detected in the five JSRV-infected lambs was 75% of the mean response detected in the four control animals. The reduced response to ConA stimulation was evident at all other time points, at six weeks the response of the JSRV infected lambs was only 38% of the response identified in the control animals, at sixteen weeks the response of the JSRV-infected lambs was 36%, in comparison with the control lambs. At eighteen and twenty weeks old two JSRV-inoculated and two control lambs remained. The mean response to ConA stimulation with the control lambs was 2212 ± 57 and 1813 ± 336 , respectively, compared with 1210 and 2059 cpm in the JSRV-inoculated lamb with no histopathologically confirmed OPA, 856 and 659 cpm for the confirmed OPA case.

3.3 Identification of PBMC subsets responding to PWM and ConA stimulation

As an altered response to ConA stimulation was detected in the JSRV-infected animals the identity of the lymphocyte subsets responding to stimulation with this mitogen were determined. For comparison, the responder cells to PWM were identified also.

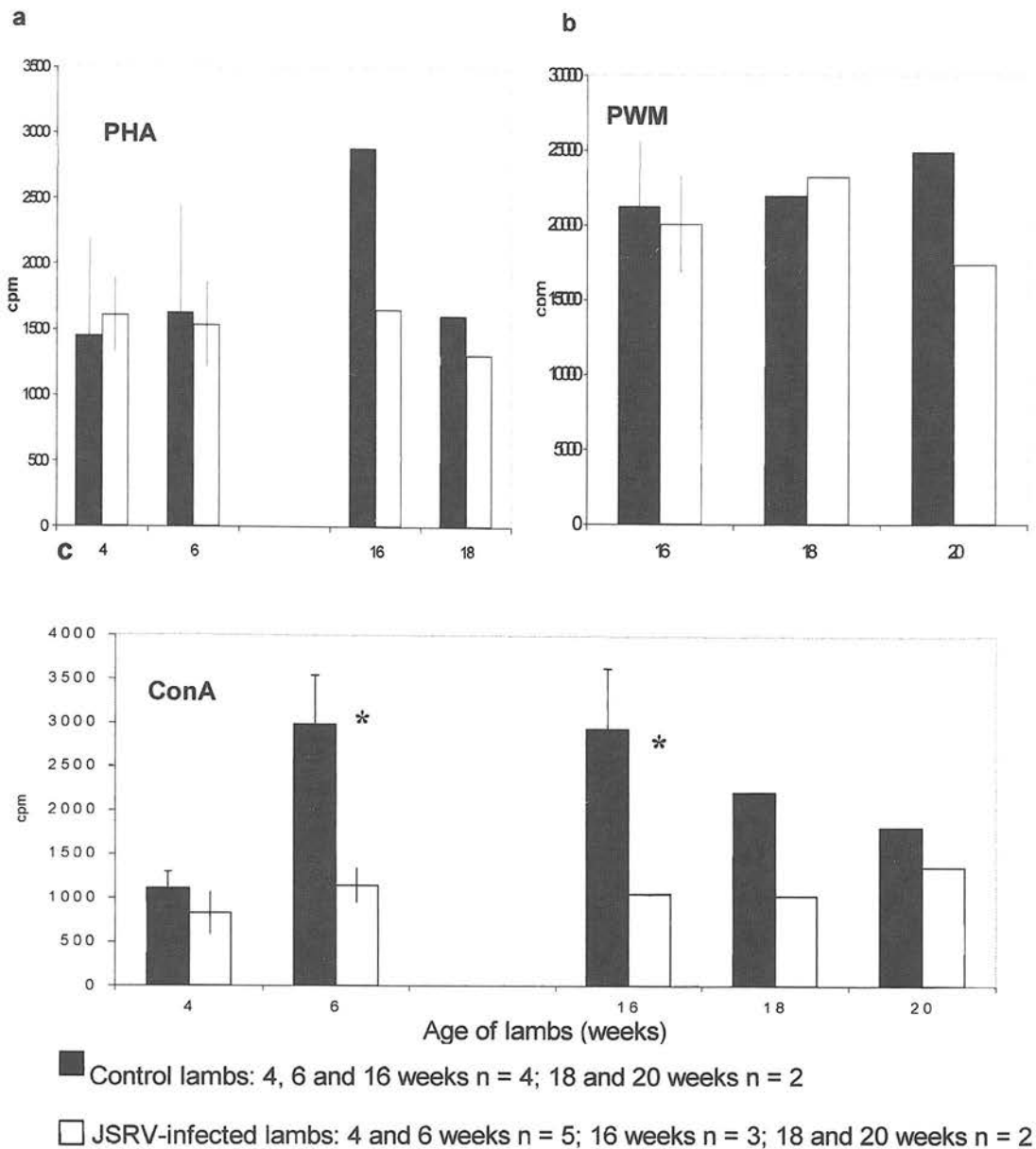


Figure 3.8. Lymphoproliferative response to PHA, PWM and ConA stimulation during experimental JSRV-induced OPA in SPF lambs. PBMC purified from the blood of SPF lambs were stimulated with PHA, PWM and ConA. The results are shown as counts per minute (cpm), \pm SEM determined by ^3H -thymidine uptake with the unstimulated PBMC cpm subtracted (4 weeks 167 : 295, 6 weeks 165 : 190, 16 weeks 224 : 229, 18 weeks 212 : 260 and 20 weeks 375 : 296 cpm) control lambs and JSRV-infected lambs respectively.. The response to PHA at 4, 6, 16 and 18 weeks is shown in Figure 3.8a. PWM at 16, 18 and 20 weeks in Figure 3.8b and to ConA at 4, 6 16 18 and 20 weeks in Figure 3.8c. No significant difference to PHA or PWM was detected between the JSRV-infected and control lambs, at any time point. In contrast statistically significantly (* $p=0.01 - 0.07$) reduced responses to ConA stimulation were detected in the JSRV-infected lambs, compared with the controls.

3.3.1 Methodology

A 20ml blood sample was collected from a two-month-old lamb (Chapter 2, section 2.1.4) and the lymphocyte subsets responding to PWM and ConA stimulation were identified by carboxyfluorescein diacetate succinimidyl ester (CFSE) - monoclonal antibody selection as detailed in Chapter 2, section 2.8, using a panel of mouse anti-sheep monoclonal antibodies specific for CD2, CD4, CD8, $\gamma\delta$ T cells and B cells.

3.3.2 T and B lymphocytes respond to PWM and ConA stimulation

PWM stimulation The CFSE results are shown in Figure 3.9 a-e, 1: scatter profile, 2: responder cell histograms, 3: table of percentage cells detected in each generation.

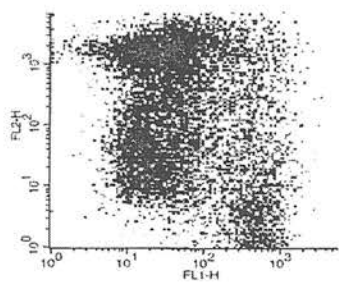
The results revealed that only 2.3% of CD2⁺ T cells remained in the parent population, and proliferation in response to PWM stimulation resulted in 27% of the CD2⁺ T cells detected in generation five, 26.5% in generation six and 18.9% in generation seven. In generation eight the percentage of CD2⁺ T cells detected was only 2.6%.

The CD2 T cell response was further characterised by CD4 and CD8 antibody labelling. The proliferative profile of CD4⁺ and CD8⁺ T cells corresponded with the response detected by CD2 monoclonal antibody selection in that both subsets responded to PWM stimulation until generation five when 28.6% CD4⁺ and 26.7% CD8⁺ T cells were detected.

The parent generation of $\gamma\delta$ T cells contained 6.9%, and 45.1% were detected in generation two. The results indicated that PWM was not strongly mitogenic for $\gamma\delta$ T cells.

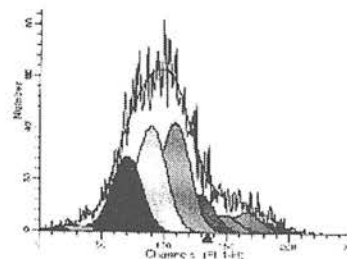
Cells responding to PWM stimulation

1

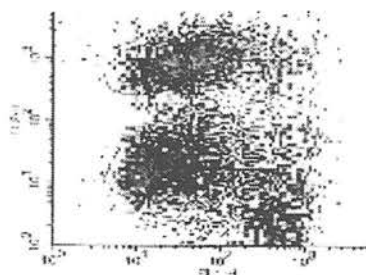


a CD2

2

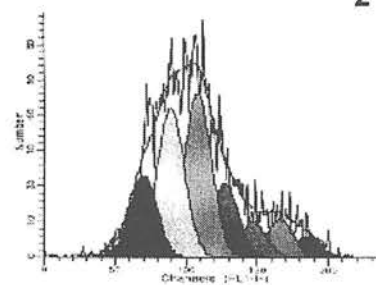


1

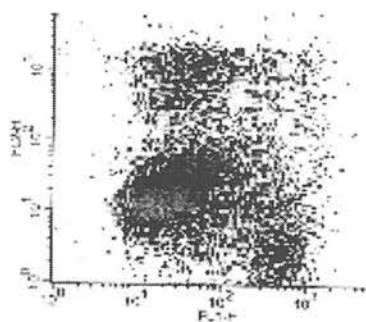


b CD4

2

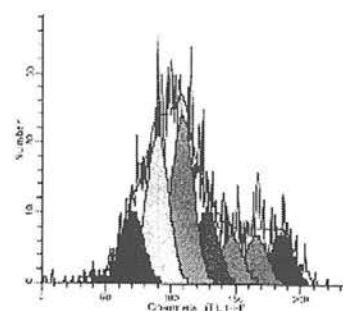


1

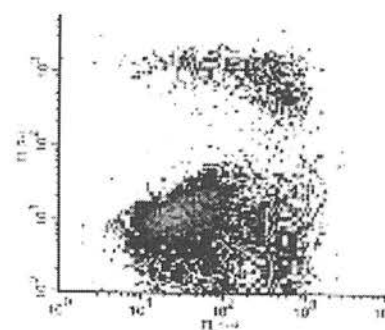


c CD8

2

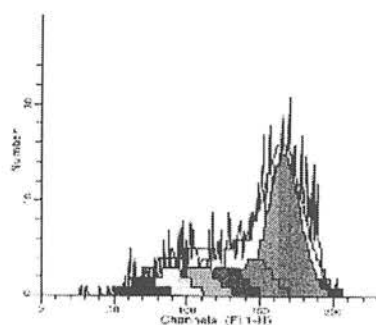


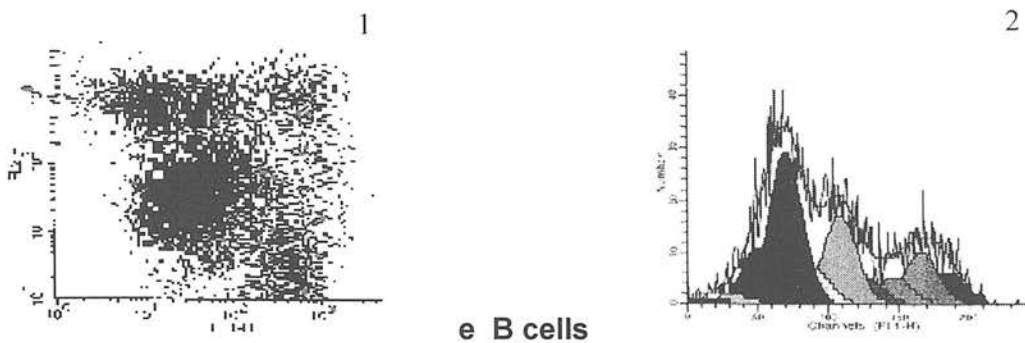
1



d $\gamma\delta$ T cells

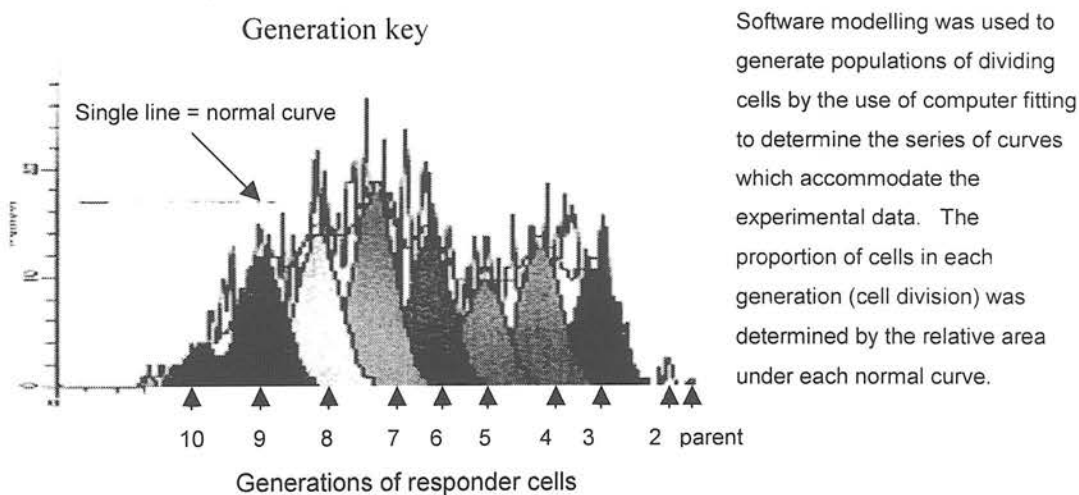
2





| 3 | Parent | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|-----------------------|--------|------|------|------|------|------|------|------|-----|-----|
| CD2 | 2.3 | 5.2 | 4.8 | 9.8 | 27.6 | 26.5 | 18.9 | 2.6 | 1.5 | 0.4 |
| CD4 | 3.8 | 6.5 | 6.2 | 12.8 | 28.6 | 26.0 | 14.3 | 1.2 | 0.2 | 0.1 |
| CD8 | 8.2 | 7.7 | 7.8 | 11.9 | 26.7 | 23.9 | 11.6 | 1.3 | 0.4 | 0.2 |
| $\gamma\delta$ T cell | 6.9 | 45.1 | 12.4 | 10.5 | 10.0 | 8.8 | 5.7 | 0.0 | 0.3 | 0.0 |
| B cells | 6.7 | 10.6 | 5.5 | 5.2 | 18.3 | 7.4 | 30.8 | 11.8 | 2.0 | 1.3 |

Figure 3.9 Carboxyfluorescein diacetate succinimidyl ester (CFSE) /monoclonal antibody detection of lymphocyte subsets responding to PWM stimulation. The PBMC were purified from the blood of a 2-month-old lamb. Prior to stimulation with PWM CFSE was incorporated into the cells. The cells were collected post-stimulation and phenotyped with a panel of monoclonal antibodies specific for CD2, CD4, CD8, $\gamma\delta$ T cells and B cells. The results are shown for each lymphocyte subset (a-e) as 1: scatter profile with responder cells, 2: histogram of generations proliferating in response to PWM and 3: table of percentage cells detected in each generation.



The results provided evidence that B cells will proliferate in response to PWM stimulation with 10.6% detected in generation two. Only approximately 5.0% B cells were found in generations three and four, with 18.3% B cells detected in generation five and 30.8% B cells detected in generation seven. The percentage of B cells in subsequent generations decreased from 11.8% in generation eight to 2% and 1.3% in generations nine and ten.

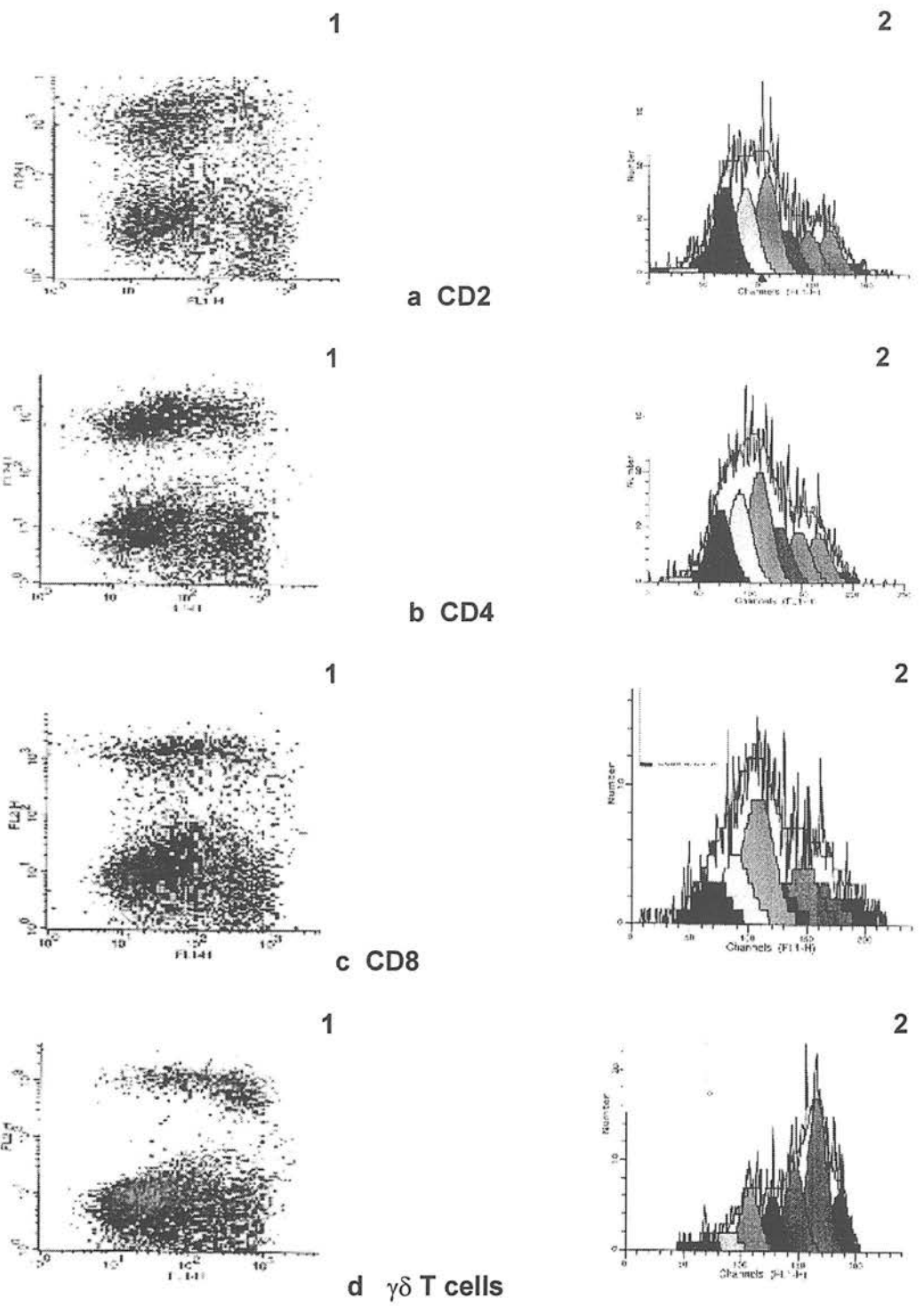
ConA stimulation The CFSE results are shown in Figure 3.10 a-e. The lymphocyte profiles in response to ConA stimulation were found to be very similar to those revealed in response to PWM. The majority of CD2 T cells responded through four generations from 2.3% detected in the parent population, with 22.7% cells detected in generation five.

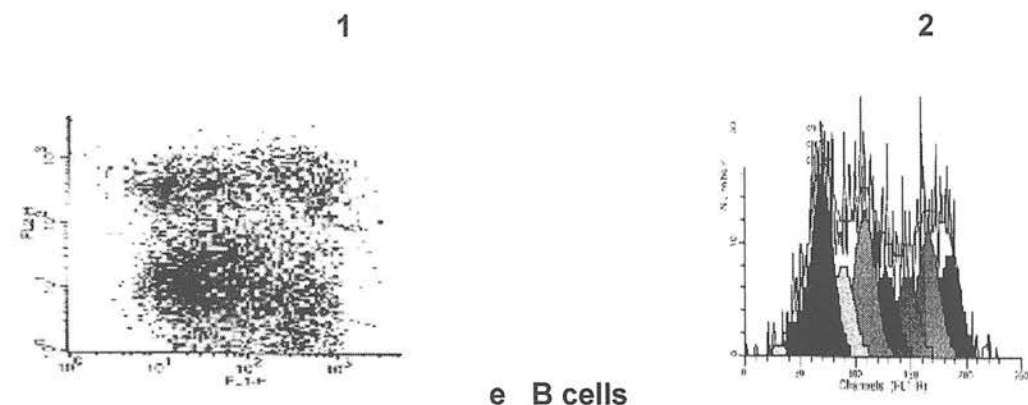
CD4 and CD8 antibody labelling revealed that CD4⁺ and CD8⁺ T cells responded to ConA stimulation. As with the response detected by CD2 monoclonal antibody selection, both subsets responded until generation five when 24.0% CD4⁺ and 30.2% CD8⁺ T cells were detected.

The $\gamma\delta$ T cell profile in response to ConA stimulation was also analogous to that identified with PWM. The parent generation of $\gamma\delta$ T cells, in response to ConA stimulation, contained 12.8%, with 33.5% detected in generation two. The percentage of $\gamma\delta$ T cells decreased from 18.7% in generations three to 3.1% detected in generation seven. Only 1.3% $\gamma\delta$ T cells were detected in generation eight and <1.0% in generations nine and ten.

B cells responded to ConA stimulation with 12.2% detected in the parent generation. The percentage of B cells detected in generations two was 14.4%, and 9.3% and 10.0% in generations three and four, respectively. The percentage of B

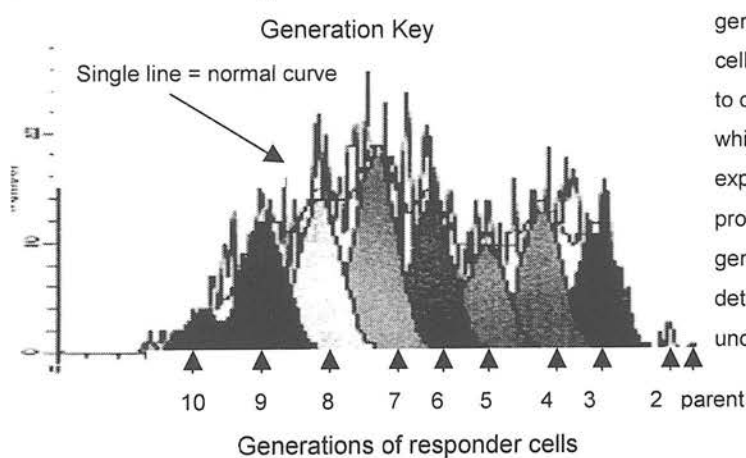
Cells responding to ConA stimulation





| 3 | parent | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|-----------------------|--------|------|------|------|------|------|------|-----|-----|-----|
| CD2 | 2.3 | 9.6 | 9.4 | 9.8 | 22.7 | 19.4 | 20.0 | 4.3 | 1.5 | 0.6 |
| CD4 | 2.9 | 10.3 | 11.0 | 12.3 | 24.0 | 19.5 | 15.9 | 2.5 | 1.0 | 0.2 |
| CD8 | 7.5 | 8.4 | 14.5 | 8.8 | 30.2 | 15.6 | 11.0 | 2.4 | 0.5 | 0.6 |
| $\gamma\delta$ T cell | 12.8 | 33.5 | 18.7 | 12.0 | 13.4 | 4.8 | 3.1 | 1.3 | 0.0 | 0.0 |
| B cells | 12.2 | 14.4 | 9.3 | 10.0 | 16.6 | 10.4 | 20.8 | 4.9 | 0.8 | 0.2 |

Figure 3.10 Carboxyfluorescein diacetate succinimidyl ester (CFSE) /monoclonal antibody detection of lymphocyte subsets responding to ConA stimulation. The PBMC were purified from the blood of a 2-month-old lamb. Prior to stimulation with ConA CFSE was incorporated into the cells. The cells were collected, post- stimulation and phenotyped with a panel of monoclonal antibodies specific for CD2, CD4, CD8, $\gamma\delta$ T cells and B cells. The results are shown for each lymphocyte subset (a-e) as 1: scatter profile with responder cells, 2: histogram of generations proliferating in response to ConA and 3: table of percentage cells detected in each generation.



Software modelling was used to generate populations of dividing cells by the use of computer fitting to determine the series of curves which accommodate the experimental data. The proportion of cells in each generation (cell division) was determined by the relative area under each normal curve.

cells found in generation five was 16.6%, with 20.8% detected in generation seven. The percentage of B cells remaining in subsequent generations decreased from 4.9% in generation eight to <1.0% in generations nine and ten.

To summarise the results: evidence of lymphoproliferation in response to PWM and ConA was demonstrated for both T and B lymphocytes. Further characterisation of the T cell population demonstrated that CD4⁺ and CD8⁺ T cell subsets responded to each mitogen. By comparison only a limited response was detected for $\gamma\delta$ T cells, as the majority of cells did not divide beyond the second generation. B cells also responded to PWM and ConA stimulation through to generation five where 18.3% (PWM) and 16.6% (ConA) were detected, but in response to each mitogen the majority of B cells progressed to generation seven with 30.8% (PWM) and 20.8% (ConA). This observation indicated that B cells do respond to PWM and ConA stimulation, and may suggest also that two B cells subsets exist in the blood of sheep, one subpopulation perhaps responding to stimulation by the mitogens directly, and a T cell directed response by a second subpopulation of B cells.

3.4 The requirement for antigen presenting cells in the lymphoproliferative response to mitogens

3.4.1 Methodology

The cells in the PBMC, which adhere following culture for one hour at 37°C, represent the major populations of antigen presenting cells. The PBMC from four lambs were purified (Chapter 2, section 2.2.1) and the lymphoproliferative responses to PHA, ConA and PWM stimulation in the entire-PBMC, the PBMC depleted of adherent cells and reconstituted-PBMC were compared (Chapter 2, section 2.5.2).

Expression of MHC class II antigens in each of the PBMC samples was determined as detailed in Chapter 2, section 2.5.3. The data were analysed and group results compiled (Chapter 2, section 2.9).

3.4.2 The lymphoproliferative response to PHA, ConA and PWM stimulation is not dependent on antigen presentation

For each mitogen tested the entire PBMC generated a higher response than the PBMC depleted of antigen presenting cells, although this difference was not found to be statistically significant (PHA $p=0.4$, PWM $p=0.4$ and ConA $p=0.2$). The response to each mitogen increased when the antigen presenting cells were returned to the PBMC. The results are shown in Figure 3.11. PHA stimulation of the entire-PBMC generated $4937 \text{ cpm} \pm 1242$. The lymphoproliferative response was reduced by 36% in the adherent-cell- depleted-PBMC. When the adherent cells were returned to the PBMC (reconstituted-PBMC) lymphoproliferation to PHA stimulation increased to 84% of the response detected in the entire-PBMC. In comparison to the response generated by the entire-PBMC ($1845 \text{ cpm} \pm 474$) to PWM stimulation, the response, after adherent cell depletion, was reduced by 36%. When the adherent cells were returned to the PBMC (reconstituted-PBMC) lymphoproliferation increased to 98% of the response detected in the entire-PBMC. The lymphoproliferative response to ConA stimulation detected with the PBMC depleted of adherent cells was reduced by 50% in comparison with the response detected in the entire-PBMC ($3225 \text{ cpm} \pm 685$). Lymphoproliferation increased by 21% when the adherent cells were returned to the PBMC.

The phenotypic profile of cells expressing MHC class II antigens in each of the PBMC samples revealed that the mean percentage of MHC class II expression was significantly reduced ($p=0.0004$) in the PBMC depleted of adherent cells

($10.6\% \pm 1$) in comparison with the entire PBMC ($32.7\% \pm 3$) and with the reconstituted PBMC ($26.9\% \pm 3$) ($p=0.002$). The results revealed however that not all antigen presenting cells were removed by adherence, and that replacement of the adherent cells to the PBMC did not fully reconstitute the entire PBMC population.

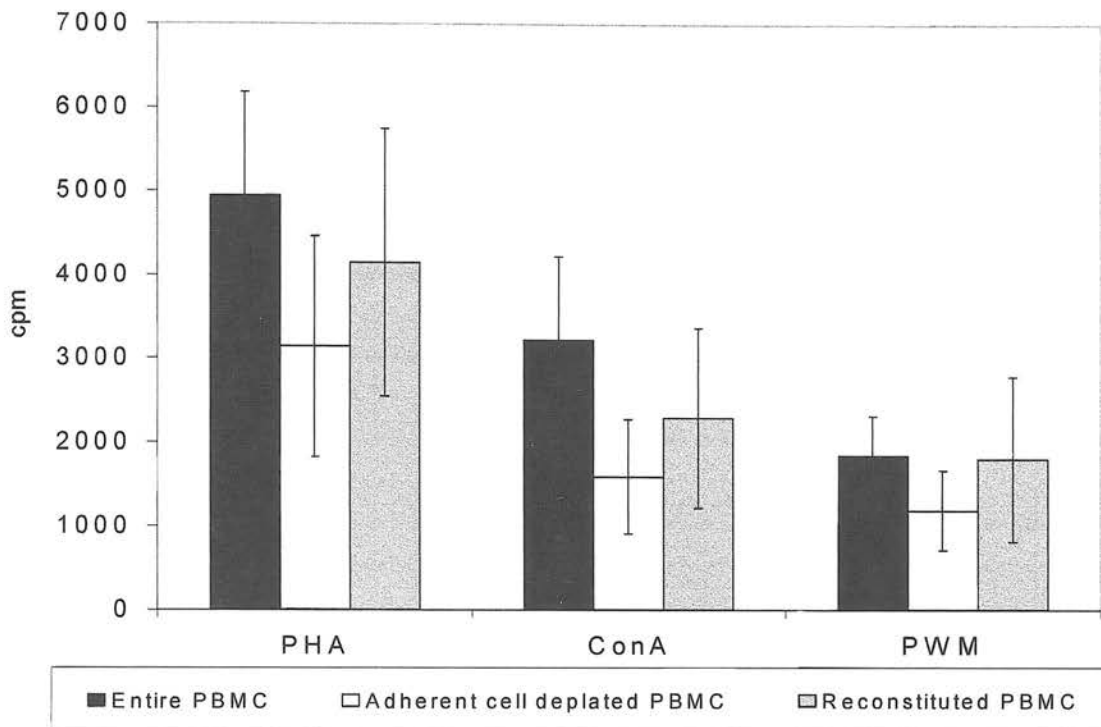


Figure 3.11 Requirement for antigen presenting, adherent cells in the mitogenicity of PHA, ConA and PWM for sheep lymphocytes. The lymphoproliferative responses to stimulation of the entire-PBMC, adherent-cell-depleted-PBMC and reconstituted-PBMC (replacement of adherent cells) were measured in blood samples collected from 4 lambs. Results are shown as counts per minute (cpm) \pm SEM, determined by ^3H -thymidine uptake uptake with the unstimulated PBMC (135 cpm) subtracted. No statistically significant difference was identified between the groups to stimulation with any of the mitogens tested.

3.5 Concentration of IFN- γ in the plasma during experimental JSRV infection.

3.5.1 Methodology

Plasma was retained from the blood samples collected at various time points during experimental JSRV infection of conventionally housed and SPF lambs. The concentration of IFN- γ detected in the blood was measured using the sandwich enzyme immunoassay, Bovine Gamma Interferon Test (BOVIGAM™) as described in Chapter 2, section 2.7. Conventionally housed lambs: four control lambs were tested from weeks one to sixteen, and two lambs at weeks eighteen and twenty. Six JSRV-infected lambs were tested from weeks one to twelve, two lambs at 18 weeks and one lamb at twenty weeks. Blood samples from two control lambs and two JSRV-infected SPF lambs were tested at all time points.

3.5.2 Experimental JSRV infection does not alter the concentration of IFN- γ detected in the blood

No significant alteration in the level of IFN- γ was detected in the blood of JSRV-infected lambs, compared with their corresponding controls, from inoculation to the onset of clinical disease.

Increased levels of IFN- γ were observed in the blood of both the infected and control conventionally housed lambs between two and eight weeks of age (Figure 3.12a). This increase coincided with an intercurrent coccidia infection in several of the animals (section 3.4.2). No detectable levels of IFN- γ were identified in the SPF lambs at any time point during the experiment (Figure 3.12b).

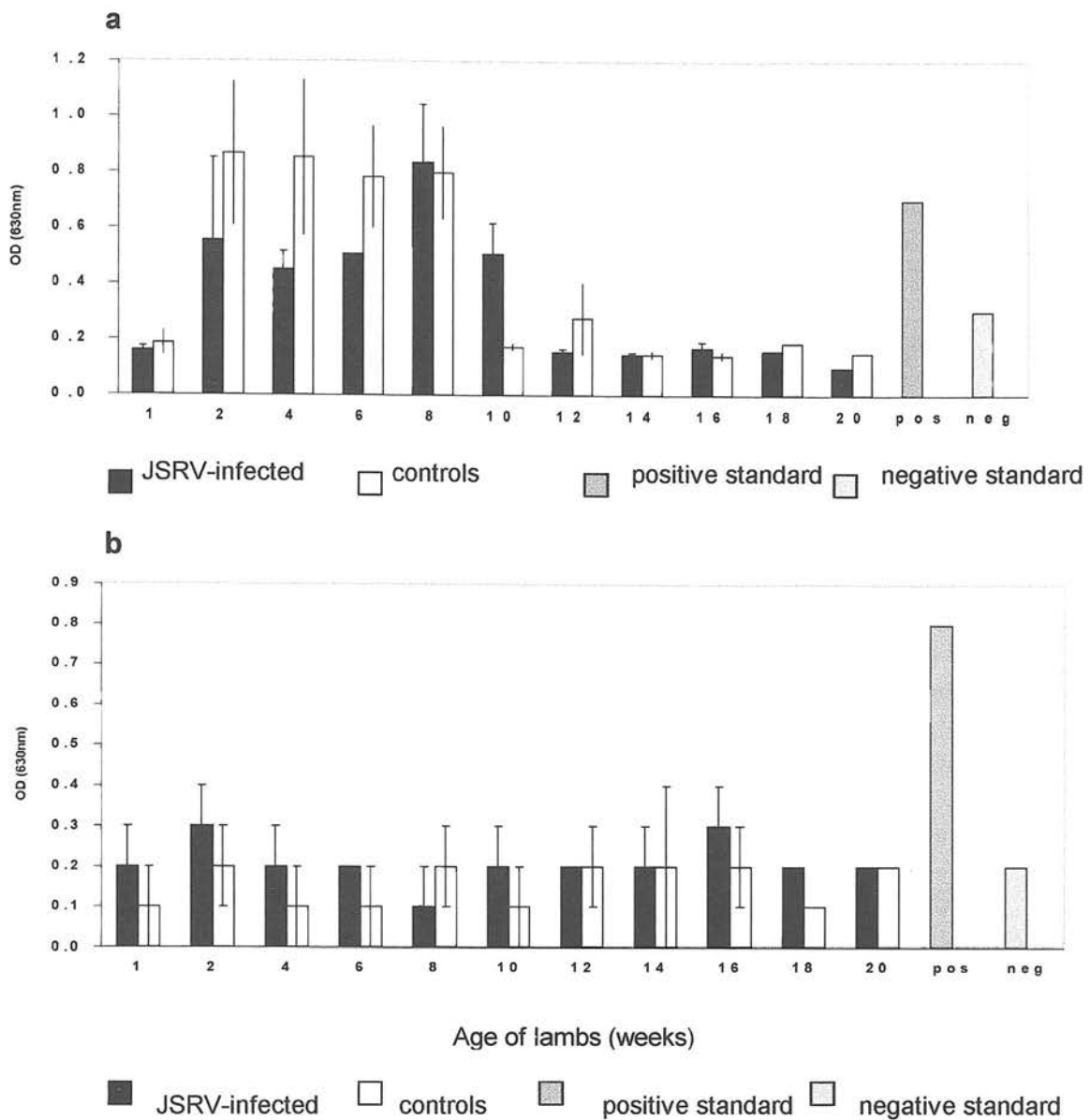


Figure 3.12 IFN- γ detected in the blood during experimental JSRV infection.

Concentration of IFN- γ measured in the plasma retained from venous blood samples collected during experimental JSRV infection of conventionally housed and SPF lambs. IFN- γ was detected by sandwich enzyme immunoassay and results determined by optical density at 630nm. The results are represented by the group mean \pm the standard deviation of the mean. Figure 3.12a: Conventionally housed control lambs: 1-16 weeks n=4, 18-20 weeks n=2. Conventionally housed JSRV-infected lambs: 1-12 weeks n=6, 14-16 weeks n=3, 18 weeks n=2, 20 weeks n=1. Figure 3.12b: SPF lambs, 2 JSRV infected and 2 uninfected control lambs at each time point.

3.6 Indirect sandwich ELISA for the detection of JSRV-capsid specific antibodies

In adult OPA cases, no circulating JSRV-specific antibodies have been recorded (Sharp and Herring 1983, Ortin *et al* 1998). It was proposed that the plasma retained from the blood samples collected during the experimental JSRV infections should be tested by indirect sandwich ELISA to measure any JSRV-specific antibodies.

3.6.1 Methodology

Plasma collected from all the lambs were tested for the presence of JSRV capsid antigen (JSRV-CA) antibodies by ELISA. A recombinant JSRV-CA coating antigen was prepared by cloning part of the JSRV *gag* gene (nucleotide sequence published by York *et al* 1992) into plasmid pBAD/His A (Invitrogen, Groningen, The Netherlands), and expressed in *E. coli* LMG 194 cells. Supernatant collected from *E. coli* culture without the CA insert was used as a negative control. Sheep anti-CA serum, as a positive control, was generated according to Palmarini *et al* (1995) with some minor adaptations. The specific absorption values were calculated by subtracting the control values from the test values. The cut-off point to define seropositivity was set as the mean OD of a negative control serum (pre-immunisation serum) plus 3 times the standard deviation of the mean (4 replicates).

3.6.2 No detectable JSRV specific antibody response was evident during experimental JSRV infection

No antibodies specific for the capsid of JSRV were found in during experimental JSRV infection and OPA development (see paper bound to this thesis). This work was performed in collaboration with Dr. Renate van der Molen (The University of Edinburgh).

3.7 Discussion

The phenotypic profile of the peripheral blood leukocytes and cell mediated responses of the systemic immune system were evaluated at all stages of disease from experimentally induced JSRV infection and tumorigenesis in neonatal lambs to the terminal stages of OPA in adult sheep. For all animals, the peripheral blood leukocytes were characterised to identify phenotypic frequency alterations and lymphoproliferation to mitogen stimulation was performed to identify any alteration in cellular function during JSRV infection. The concentration of IFN- γ in the blood of experimental lambs was measured also. The results of each study are summarised in Figure 3.13.

This investigation of the systemic immune responses, following experimental JSRV infection, has identified for the first time an alteration in cellular function. A significantly reduced response to ConA stimulation was evident in the PBMC from the blood of conventionally housed and SPF, JSRV-infected lambs (section 3.2.5.2). Furthermore, the suppressed response to ConA stimulation was detected as an early event, as it was first apparent from eight weeks of age, prior to the diagnosis of clinical disease. Moreover, the change in cellular function was evident in natural JSRV infection also, demonstrated by the reduced response to ConA stimulation in adult sheep, in the terminal stages of OPA (section 3.1.2.2). No difference between the JSRV-infected and control animals was detected when the PBMC were stimulated with PHA and PWM. It is widely known that there is no common carbohydrate specificity between the three lectins; PHA binding to glycoproteins containing N-acetylgalactosamine, PWM binding to

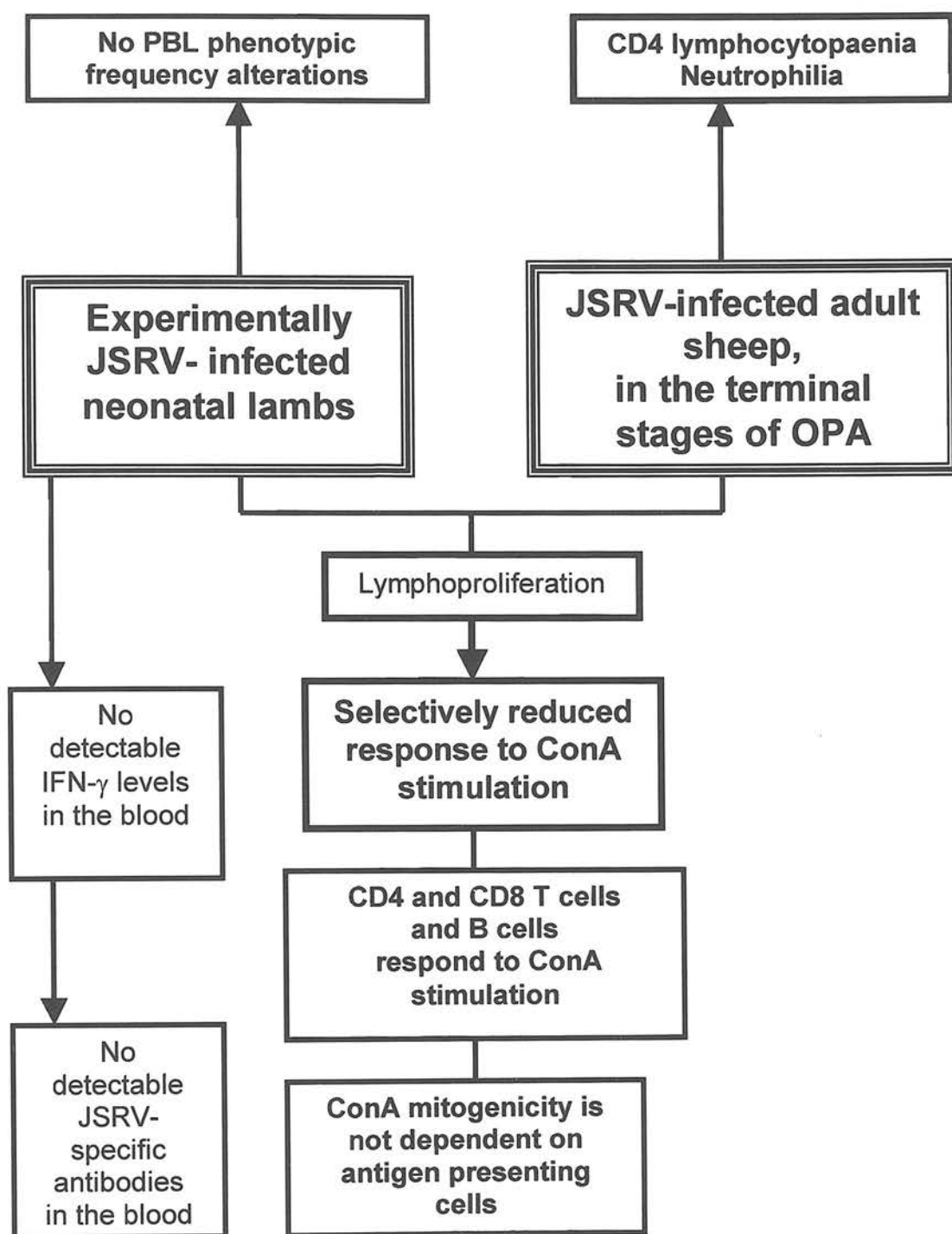


Figure 3.13 Summary of results: Systemic immunity during JSRV infection

N-acetylglucosamine and ConA having an affinity for α -D-mannosyl and α -D-glucosyl residues.

The cells responding to PWM and ConA stimulation were identified by CFSE/monoclonal antibody immunolabelling and the types of cells responding to ConA stimulation did not differ from the cells responding to PWM (section 3.3). Both CD4 and CD8 T cells were induced to undergo mitotic division, and only a limited response was detected for $\gamma\delta$ T cells. CFSE identification of responder cells indicated that PWM and ConA stimulation induced B cell proliferation that is possibly T cell dependant. This may suggest that the activation of T cells and their ability to induce B cell lymphoproliferation could be impaired by the presence of the virus.

The reason for the reduced response to ConA stimulation is as yet unknown but several factors, identified in other retroviral infections, indicate a possible mechanism for this observation during JSRV infection. The envelope proteins of human immunodeficiency virus (HIV) (Pahwa *et al* 1986), human T cell leukaemia virus (HTLV) (Wainberg *et al* 1985), baboon leukaemia virus (BaLV) (Denner *et al* 1990) and Mason-Pfizer monkey virus (MPMV) (Blaise *et al* 2001) are all capable of suppressing lymphoproliferative responses to mitogen stimulation. It has been established that retroviral suppression can be selective for one mitogen without the response to other mitogens being affected. For example, HIV-1 inhibits PHA induced lymphoproliferation but does not affect the response to ConA stimulation (Mann *et al* 1987). The transmembrane protein p15E from FeLV (Mathes *et al* 1978, 1979) or MuLV (Ruegg *et al* 1989, Schmidt *et al* 1987) induces immunosuppression by preventing aggregation of ConA receptors, suggesting that expression of this

protein interferes with the redistribution of the cytoskeleton (Dunlap *et al* 1979). Therefore, in JSRV infection and OPA development, specific changes in glycoprotein-bound-carbohydrate residues resulting from the presence of JSRV may be involved.

To test the hypothesis that the mitogenicity of ConA may depend on antigen presentation, the PBMC were depleted of adherent cells. However, it was demonstrated in this study that the depletion of adherent cells did not significantly affect the lymphoproliferative response to ConA, or indeed to PHA or PWM stimulation. However, it must be noted that the removal of adherent cells did result in a decrease in the lymphoproliferative response (particularly to ConA), and the requirement for antigen presentation was only tested in uninfected lambs. As the removal of adherent cells did not result in total depletion (identified by MHC class II immunolabelling), the few remaining cells might be sufficient for antigen presentation. Furthermore, adherence does not remove the (non-adherent) B cells, which were retained within the PBMC. Therefore this preliminary result is not conclusive (section 3.4.2).

During the disseminated JSRV infection, the highest level of proviral load (1 infected cell per 1,524 cells) was identified in the monocyte/macrophage population (Holland *et al* 1999). Retrovirally-induced cytokine alterations resulting in down-regulation of lymphocyte and monocyte functions have been demonstrated. Reduced levels of IL-2, IFN- α and IFN- γ in the presence of FeLV resulted in suppressed lymphoproliferative response to ConA stimulation (Hebebrand *et al* 1977, Copelan *et al* 1987). Decreased IFN- γ , paralleled by increased levels of IL-10, impeding the ability of monocytes to promote IFN- γ production by T cells (Haraguchi *et al* 1995),

and retrovirus-induced cytokine switching from IL-12 to IL-4 in dendritic cells has also been identified; IL-12 being essential for dendritic cell maturation into antigen presenting cells capable of stimulating T cell proliferation (Kellecher *et al* 1999). In this investigation no alterations in IFN- γ levels, specific to infected animals, were detected throughout the experiments (section 3.5.2). Consequently during experimental JSRV infection there was no evidence to suggest that retrovirally induced cytokine switching had occurred involving IFN- γ . Furthermore no JSRV specific antibodies were detected at any time point during the experimental infection.

The phenotypic profile of the peripheral blood leukocytes were monitored during experimental JSRV infection. As a consequence of the data generated, the development of the ovine systemic immune system in young lambs was demonstrated. The phenotypic frequencies of T cells, B cells and neutrophils in the blood of the conventionally housed lambs were measured pre-JSRV inoculation, at weekly intervals (except week 3) until ten weeks old and subsequently at two-week intervals until the end of the experiment at twenty weeks. Phenotypic monitoring was repeated with the SPF control lambs pre-inoculation and at two-week intervals until twenty weeks. Blood samples were collected from four conventionally housed control lambs and four SPF control lambs until sixteen weeks, and from two lambs in each group at eighteen and twenty weeks.

Development of the systemic immune system of the conventionally housed lambs is shown in Figure 3.14a. For the first two weeks after birth a higher percentage of neutrophils (week 1: $27\% \pm 8$, week 2: $23\% \pm 6$) was detected in the blood, in comparison with the percentage of positively immunolabelled lymphocytes detected (T cells: week 1: $4\% \pm 1$, week 2: $15\% \pm 1$, B cells week 1: $1\% \pm 1$, week

2: $7\% \pm 1$). When the lambs were four weeks of age, the percentage of immunolabelled T ($19\% \pm 3$) and B lymphocytes ($12\% \pm 2$) in the blood were higher than the percentage of neutrophils ($8\% \pm 2$). The percentage of immunolabelled T and B cells continued to increase, with no significant alteration in the percentage of neutrophils detected in the blood, until the lambs were twenty weeks old. It was observed that the percentage of immunolabelled B cells in the blood ($25\% \pm 2$) was higher than the percentage of T cells ($20\% \pm 5$) at five weeks old, corresponding to the time when mastitis was diagnosed in two of the dams (section 3.4.1); again in comparison to the percentage of immunolabelled T cells, equivalent or higher percentages of immunolabelled B cells were detected in the blood when the lambs were seven and eight weeks old, possibly in response to the coccidia infection diagnosed in several of the lambs.

Development of systemic immunity in the SPF lambs is shown in Figure 3.14b. Pre-JSRV inoculation a higher percentage of neutrophils ($17\% \pm 6$) were detected in the blood, in comparison with the percentage of positively immunolabelled lymphocytes (T cells: $11\% \pm 4$, B cells $3\% \pm 1$). When the lambs were one week old, the percentage of positively immunolabelled T cells ($17\% \pm 5$) was higher than the percentage of neutrophils ($12\% \pm 1$), with $6\% \pm 2$ B cells detected and at two weeks the percentage of immunolabelled T cells ($24\% \pm 5$) and B cells ($10\% \pm 2$) in the blood had increased, in comparison with the percentage of neutrophils ($13\% \pm 6$). From four weeks of age, until the lambs were twenty weeks old the percentage of immunolabelled T and B cells detected in the blood continued to increase, with no significant alteration in the percentage of neutrophils detected.

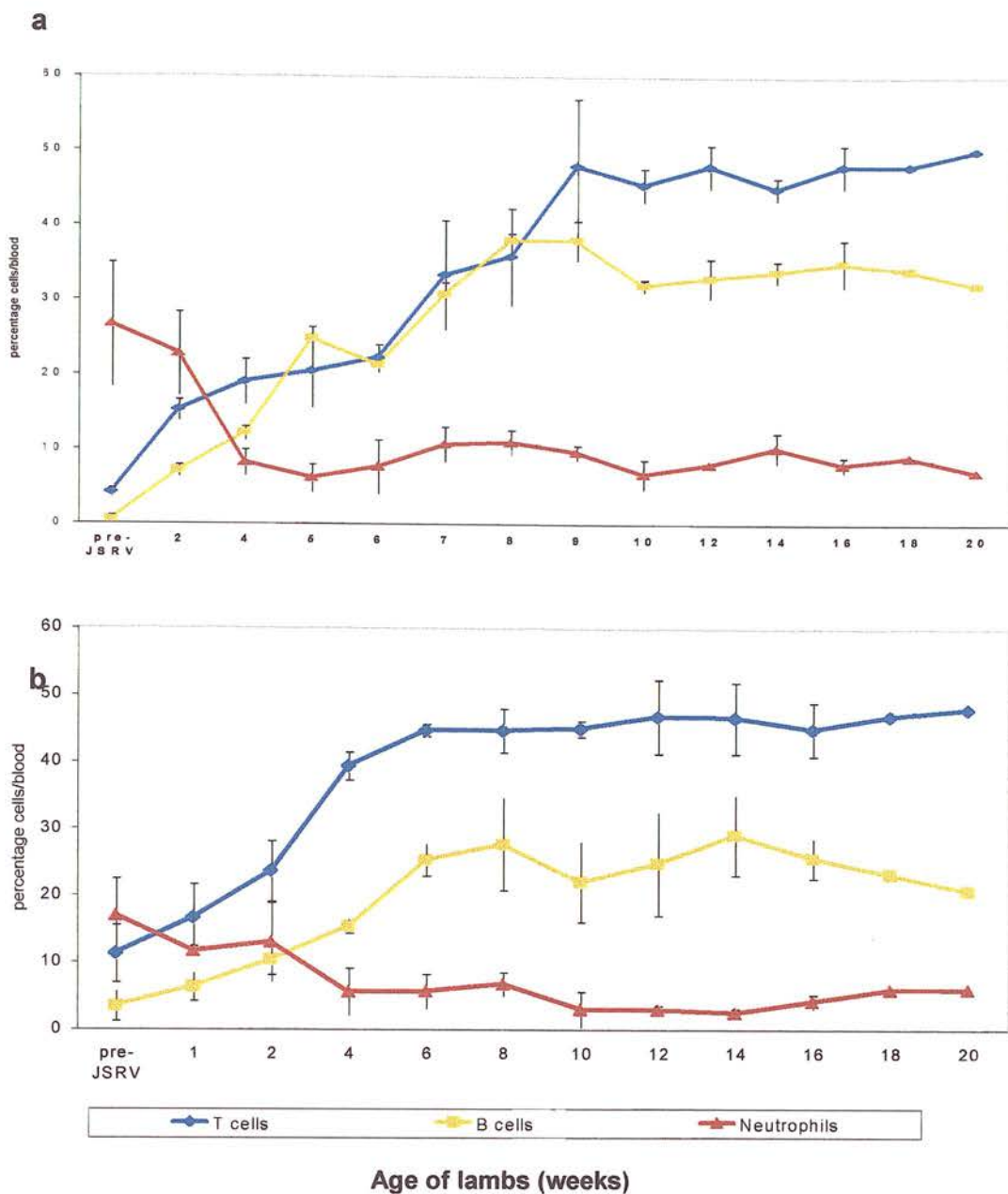


Figure 3.14 Ontogeny of ovine systemic immunity. The PBMC purified from the blood of the control lambs were phenotyped with a monoclonal antibody specific for CD2T cells and B cells. The results were determined by flow cytometry and are shown as the percentage of positively immunolabelled cells/blood. The neutrophils were counted also. Each time point represents the group mean \pm SEM. Figure 3.7a: The conventionally housed lambs, 1-16 weeks $n=4$, 18-20 weeks $n=2$. Figure 3.7b: SPF lambs, 1-16 weeks $n=4$, 18-20 weeks $n=2$.

A T cell : B cell mean ratio of 2 : 1 within the range 1.6 to 2.8, was demonstrated for lambs aged four to twenty weeks.

The observed trend could indicate that innate immunity (determined by the percentage neutrophil population in the blood) predominated in both conventionally housed and SPF neonatal lambs until the acquired immune system (determined by the increasing percentage of positively immunolabelled T and B lymphocytes) became fully established from four weeks of age. It is not suggested, however, that the lymphocytes were absent from the blood at an early age, but may indicate that immature lymphocytes do not express the cell surface receptors for the monoclonal antibodies used.

This present study revealed no alteration in phenotype frequencies in the peripheral blood throughout experimental JSRV infection (section 3.2.3.2). The CD4⁺ lymphocytopaenia detected in the blood of adult sheep during the terminal stages of OPA, by Rosadio and Sharp (1992), Holland *et al* (1999) and in this study was not identified during experimental JSRV infection. Similarly the neutrophilia, demonstrated here and by Rosadio and Sharp (1992) in the blood of adult OPA cases was not observed during experimentally-induced tumorigenesis (section 3.1.1.3). Until tests are developed to diagnose JSRV infection prior to the onset of clinical symptoms, it is not possible to detect when, during natural infection, the lymphocytopaenia and neutrophilia first occur. However, the results obtained in this study provide evidence that the lymphocytopaenia and neutrophilia are not early events in JSRV infection and suggest that they might not be a direct response to JSRV infection but a consequence of increased susceptibility to secondary infections, encountered as the health of the animal declines.

Chapter 4

The mitogenicity of the monocotyledenous *Narcissus pseudonarcissus* lectin

Introduction

Since the mitogenic properties of plant lectins were first identified by Nowell in 1960, their ability to induce non-specific lymphoproliferation has been used as a standard laboratory tool to evaluate immunocompetence (Lis and Sharon 1977 and 1986). By inducing mitotic division in resting lymphocytes, activated, cytokine-producing cells are generated, allowing *in vitro* evaluation of immune status, alteration in cellular function and cell-mediated responses during infection (Barker 1969, Kilpatrick 1998). Lectin-induced mitogenicity has been demonstrated in T cells, both CD4 and CD8 subsets, and B cells, with divergence to various maturation stages, including fully differentiated plasma cells (Kilpatrick 1998 and 1999).

Most of the commercially available lectins are derived from dicotyledonous plants such as phytohemagglutinin (PHA) from the red kidney bean *Phaseolus vulgaris*, concanavalin A (ConA) from the jack bean *Canavalia ensiformis* and pokeweed mitogen (PWM) from the poke plant *Phytolacca americana*. The biological activity of such lectins are well documented (Kilpatrick 1991 and 1995).

The mitogenicity of lectins derived from monocotyledonous sources, in particular members of the grass family such as wheat germ agglutinin (WGA), also have been extensively examined (Kilpatrick and McCurrach 1987). Lectins from several non-grass monocotyledonous plants have been isolated recently and their properties evaluated (Kilpatrick *et al* 1999). Several species of plants from the superfamily *Amaryllidaceae* possess lectins that are specific for the carbohydrate mannose (Van Damme *et al* 1988). One such lectin is *Narcissus pseudonarcissus* agglutinin (NPA) derived from daffodil bulbs; NPA exists as a non-glycosylated dimeric or trimeric structure composed of 12.5kDa subunits with three binding pockets per

monomer, specific for α 1-3 linked mannose and α 1-6 mannose residues (Van Damme *et al* 1988). A study by Sauerborn *et al* (1999) has identified NPA in a tetrameric form, suggesting that this lectin could possess potentially up to 12 binding pockets per cluster.

It has been proposed that the specificity of NPA for mannose could be exploited to combat retroviral infections, where the viruses or viral components contain glycoproteins with terminal α 1-3 linked or α 1-6 linked D-mannose. It has been reported that the infectivity of human immunodeficiency virus (HIV) types 1 and 2 (Muller *et al* 1988 and Balzarini *et al* 1991), and simian immunodeficiency virus (SIV) (Weiler *et al* 1990) have been attenuated in the presence of mannose-binding-lectins, by direct competitive inhibition. Hence the development of several potentially antiretroviral strategies based on the mannose specificity of NPA have been proposed, for example the development of NPA-based-medication, and also a vaccine against RNA viruses, encompassing anti-lectin antibodies, consequentially targeting the virus indirectly. This strategy is possibly applicable to OPA, as no JSRV-specific antibodies have been detected in the blood of infected animals (Sharp and Herring 1983, Ortin *et al* 1998). Furthermore, the use of NPA, in conjunction with radioisotopes, fluorochromes or enzymic labelling, as diagnostic tools in the detection and *in situ* identification of retroviruses is also under consideration (Scottish Crop Research Institute, European Patent 0497825, 1996).

With the potential use of NPA a possibility in JSRV research, the principal aim of the present study was to assess the mitogenicity of NPA for sheep lymphocytes, and ascertain if the lymphoproliferative response to NPA stimulation was altered in JSRV-infected compared with uninfected sheep.

Optimum NPA concentration As the lymphoproliferative response to this lectin was unknown in sheep, the optimum concentration of NPA required to induce maximum proliferation was determined by stimulating the PBMC purified from blood samples of adult sheep, and lambs aged one month. The optimum concentration of NPA was determined from the resulting standard curves (section 4.1.1).

Determination of age-dependency in the mitogenicity of NPA for sheep lymphocytes

As a consequence of the unexpected findings disclosed during the NPA optimisation assays, it was suspected that NPA could be mitogenic for the PBMC from young lambs and not adult sheep. An investigation to determine age-related lymphoproliferation to NPA stimulation was conducted with lambs aged four days to four months of age, and also adult sheep aged one to three years (section 4.1.2).

Identification of PBMC subsets responding to NPA stimulation The aim of this study was to determine which lymphocyte subset(s) responded to NPA stimulation. Dual colour flow cytometry, utilising internalised carboxyfluorescein diacetate succinimidyl ester (CFSE) and monoclonal antibody selection (Chapter 2, section 2.8) was performed to phenotypically characterise the generations of cells proliferating in response to NPA stimulation (section 4.2).

The dependence of NPA mitogenicity on antigen presenting cells To ascertain if the mitogenicity of NPA required antigen presenting cells, the lymphoproliferative response to NPA stimulation was measured and compared in the entire-PBMC population, adherent cell-depleted-PBMC and reconstituted-PBMC (adherent cells replaced), all derived from the same blood sample (section 4.3).

The mitogenicity of NPA during JSRV infection To identify any alteration in lymphoproliferation in the presence of JSRV, the response to NPA stimulation was

measured and compared in the PBMC from experimentally JSRV-infected and control lambs (section 4.4.1).

Comparison of the lymphocyte phenotypic profile, pre-and-post-NPA stimulation

This comparative study was performed to identify any phenotypic frequency alterations, post-NPA stimulation, in JSRV infected and control lambs. Before and after NPA stimulation, the PBMC of JSRV-infected and control lambs were phenotyped by labelling with monoclonal antibodies recognising T and B lymphocytes, and the results determined by flow cytometry. The lymphocyte profiles identified for the individual lambs, pre-and-post-stimulation, were compared. Furthermore, the profiles from the JSRV-infected group were compared with those detected in the control group (section 4.4.2).

Mannose Inhibition of NPA mitogenicity It has been established that NPA is specific for α 1-3 linked and α 1-6 linked mannose. Consequently, providing mitogenicity occurs through the mannose pathway, it was hypothesised that the addition of exogenous mannose to the lectin, prior to incubation with the PBMC, should suppress the lymphoproliferative response by competitive inhibition. Therefore, to determine if NPA mitogenicity for the PBMC of lambs was *via* the mannose pathway, the response to NPA following pre-incubation with mannose was measured (section 4.5).

Optimum mannose concentration The optimum concentration of mannose required to achieve the maximum level of inhibition to NPA stimulation was measured and compared with the mitogenicity of NPA alone. (section 4.5.1.2).

Mannose inhibition during JSRV infection To determine if the inhibitory effect of mannose on NPA mitogenicity was affected by the presence of JSRV, comparative analysis of JSRV-infected and control lambs was performed (section 4.5.2).

Determination of age-dependency on the inhibitory effect of mannose It was envisaged that the level of mannose inhibition on NPA mitogenicity may be related not only to the presence of JSRV but also to the age of the lambs. Therefore the lymphoproliferative responses to NPA and NPA/mannose stimulation were measured, at various ages, in JSRV-infected and control lambs. (section 4.5.2).

NPA mitogenicity for human lymphocytes The investigation into the mitogenicity of NPA for sheep PBMC unexpectedly revealed that NPA was mitogenic in lambs and not adult sheep (section 5.1.2). The significance of this finding may have far reaching consequences for humans and domesticated animals, as interest in monocotyledonous lectins has evolved since the discovery that several members of the super-family *Amaryllidaceae*, such as NPA and *Galanthus nivalis* (GNA) derived from snowdrop bulbs have an antimetabolic effect on insects (Down *et al* 1996). It was hypothesised that the insecticidal properties could be harnessed to transgenically modify arable crop plants. Consequently several prototype crops have already been developed (Machuka *et al* 1999). It was envisaged that NPA, identified as virtually non-mitogenic for adult humans (Kilpatrick *et al* 1990, Fenton *et al* 1999) may be mitogenic for the lymphocytes of children, therefore a comparative investigation to measure the human lymphoproliferative response to NPA stimulation was undertaken (section 4.6).

Optimum NPA concentration The optimum concentration of NPA required to induce maximum proliferation in human lymphocytes was determined by stimulating the

mononuclear cells purified from umbilical cord blood, and the PBMC from adult venous blood.. The optimum concentration of NPA was determined from the resulting standard curves (section 4.6.1.2).

Determination of age-dependency in the mitogenicity of NPA for human

lymphocytes A comparative study measuring the lymphoproliferative response to NPA stimulation, with mononuclear cells from umbilical cord blood and adult PBMC, was performed (section 4.6.1.3).

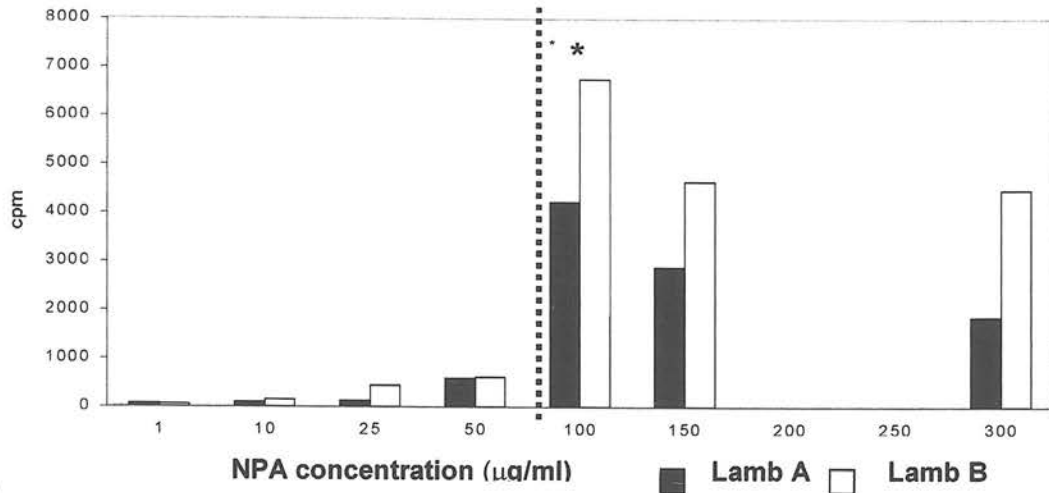
4.1 Sheep lymphoproliferative response to NPA stimulation

4.1.1 Optimisation of NPA concentration

The PBMC were purified from blood samples collected from two, one-month-old lambs as detailed in Chapter 2, section 2.2.1 and adjusted to a final concentration of 10^6 PBMC/ml. The proliferative response to stimulation with various concentrations of NPA (Vector Laboratories, Peterborough, England) was determined, following the lymphoproliferation protocol in Chapter 2, section 2.5. The data were analysed as detailed in Chapter 2, section 2.9. The optimum concentration of NPA determined from the titration series shown in Figure 4.1a was found to be 100 μ g/ml, generating 4228 counts per minute (cpm) for lamb A and 6756 cpm for lamb B. To determine if this concentration of NPA was optimum for adult sheep also, lymphoproliferation was performed as detailed above with the PBMC purified from the blood of two adult animals (Chapter 2, section 2.2). The titration series produced is shown in Figure 4.1b. No lymphoproliferative response was detected with any of the concentrations of NPA previously tested with the PBMC from the lambs, the maximum response (after subtraction of the un-

stimulated control PBMC) was <100cpm. Therefore the concentration series was extended up to 800 μ g/ml. The results revealed unexpectedly that NPA was non-mitogenic for the PBMC from adult sheep, at all the concentrations tested.

a



b

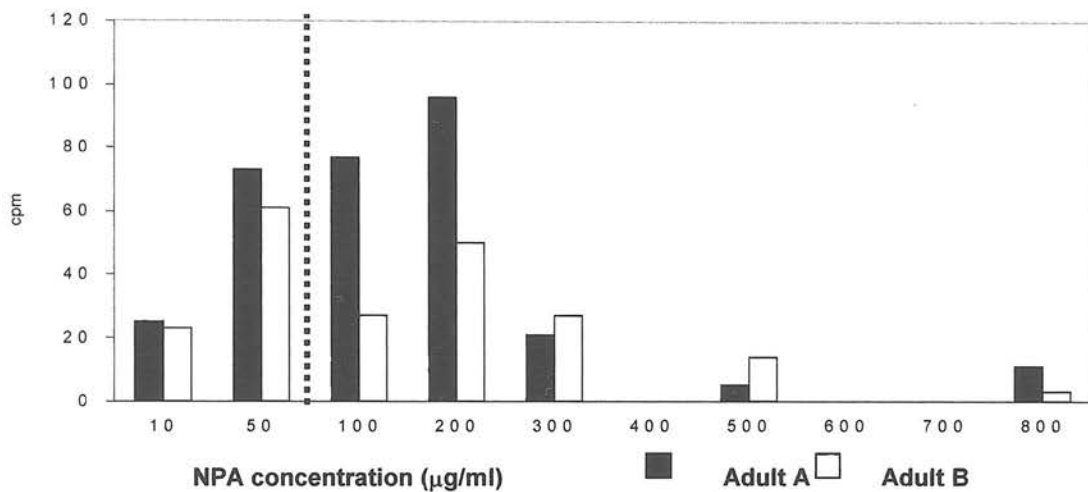


Figure 4.1 Optimisation of NPA concentration. Titration series to determine the optimum concentration of NPA required to induce maximum lymphoproliferation in sheep PBMC. The results are shown as counts per minute (cpm), determined by 3 H-thymidine uptake. Figure 4.1a: The PBMC from two 1-month-old lambs were stimulated with various concentrations of NPA. The cpm from unstimulated PBMC (lamb A 181; lamb B 212 cpm) were subtracted. The maximum lymphoproliferative response to NPA stimulation was detected *100 μ g/ml. Figure 4.1b: The PBMC from two adult sheep were stimulated with various concentrations of NPA. The cpm from unstimulated PBMC (sheep A 221; sheep B 243 cpm) were subtracted. No lymphoproliferative response to NPA stimulation was detected in adult sheep, at any concentration tested.

4.1.2 NPA stimulation of the PBMC from lambs and adult sheep

4.1.2.1 Methodology

To extend the findings that NPA is non-mitogenic for the PBMC from adult sheep, an investigation into age-related mitogenicity was performed. In total, the PBMC were purified from 74 blood samples. The animals were categorised into three groups: Group A, lambs aged four days to one month ($n = 37$), Group B, lambs aged two – four months ($n = 26$), and Group C, adult sheep aged one – three years ($n = 11$). The lymphoproliferative response to PHA and NPA stimulation was determined for the three age groups. The data were analysed and group results compiled (Chapter 2, section 2.9).

4.1.2.2 NPA mitogenicity is age-related in sheep

In response to PHA stimulation no significant difference was detected in the mean counts per minute (cpm) between the three age groups (group A 9039 ± 4226 , group B 8906 ± 5076 and group C 8198 ± 1524). In contrast, stimulation with NPA generated a mean cpm of 5154 ± 445 and 2203 ± 616 for the lambs Groups A and B, respectively, in comparison to a mean cpm of 330 ± 128 for the adult Group C. The results obtained for the lambs, in comparison with the adult sheep were found to be statistically significantly higher at $p = 0.0005$ for group A and $p = 0.04$ for group B (Figure 4.2).

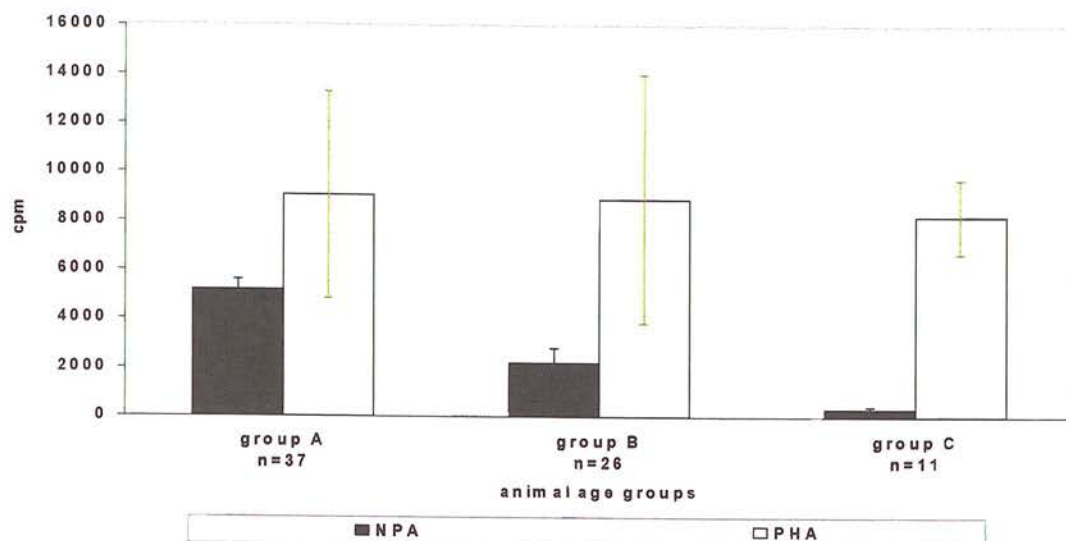


Figure 4.2 Age-related lymphoproliferative response to NPA and PHA stimulation. The PBMC of 74 animals (categorised in 3 age groups, A B and C) were stimulated with NPA and PHA. The results are shown as counts per minute (cpm) \pm SEM, determined by ^3H -thymidine uptake with the unstimulated PBMC cpm (group A 152; group B 284; group C 184 cpm) subtracted. Comparison in the levels of lymphoproliferation between the 2 groups of lambs (A and B) and the adult group C were not significantly different in response to PHA stimulation, but were found statistically significant in response to NPA, group A ($p = 0.0005$) and group B ($p = 0.004$).

4.2 Identification of the PBMC subsets responding to NPA stimulation

4.2.1 Identification of lymphocyte subsets expressing cell surface receptors for NPA

To determine which lymphocyte subsets present in sheep peripheral blood possess a receptor for NPA, the PBMC were incubated with NPA conjugated to the fluorochrome, fluorescein (NPA/FITC) (Vector Laboratories, Peterborough, England) and counter-labelled with mouse anti-sheep monoclonal antibodies specific for CD2, CD4, CD8, $\gamma\delta$ T cells and B cells, followed by incubation with a rabbit

anti-mouse immunoglobulin/phycoerythrin conjugate (Ig/PE) (Sigma, Poole, Dorset).

The PBMC subsets binding the lectin were identified by dual colour flow cytometry.

4.2.1.1 Methodology

The PBMC were purified (Chapter 2, section 2.2) from the blood of a two month old lamb. Aliquots of 5×10^5 PBMC were incubated with NPA/FITC for 30 minutes at room temperature, in the dark. After incubation 1ml PBS was added to each aliquot and centrifuged for 5 minutes at 300g. The supernatant was decanted and the PBMC were immunolabelled (Chapter 2, section 2.3) with the panel of primary monoclonal antibodies detailed above and a mouse IgG control. The secondary antibody Ig/PE was added to each sample (100 μ g/ml), replacing the Ig/FITC detailed in Materials and Methods. The results were determined by dual colour flow cytometry, using an EPICS XL-MCL™ profile (Coulter Electronics, Miami, Florida, USA), utilizing argon ion lasers set at wavelengths of 488nm (FL-1: FITC absorbs blue light and emits green fluorescence) and 600nm (FL-2: PE absorbs yellow-green light and emits red fluorescence). The percentage of double-labelled cells for each of the PBMC subsets was identified by dual fluorescence (FL1/FL2) quadrant separation, with colour compensation adjusted to eliminate dual colour autofluorescence. The background control was set at 1.0% and data were collected on a minimum of 5000 lymphocytes.

4.2.1.2 T and B lymphocytes possess the receptor for NPA

All the lymphocyte subsets analysed were found to express cell surface receptors for NPA, identified as the percentages of double-labelled cells in the blood. The results for each subset are shown in Figure 4.3, with the exception of the CD2 immunolabelled sample, which was accidentally deleted from the computer, but the

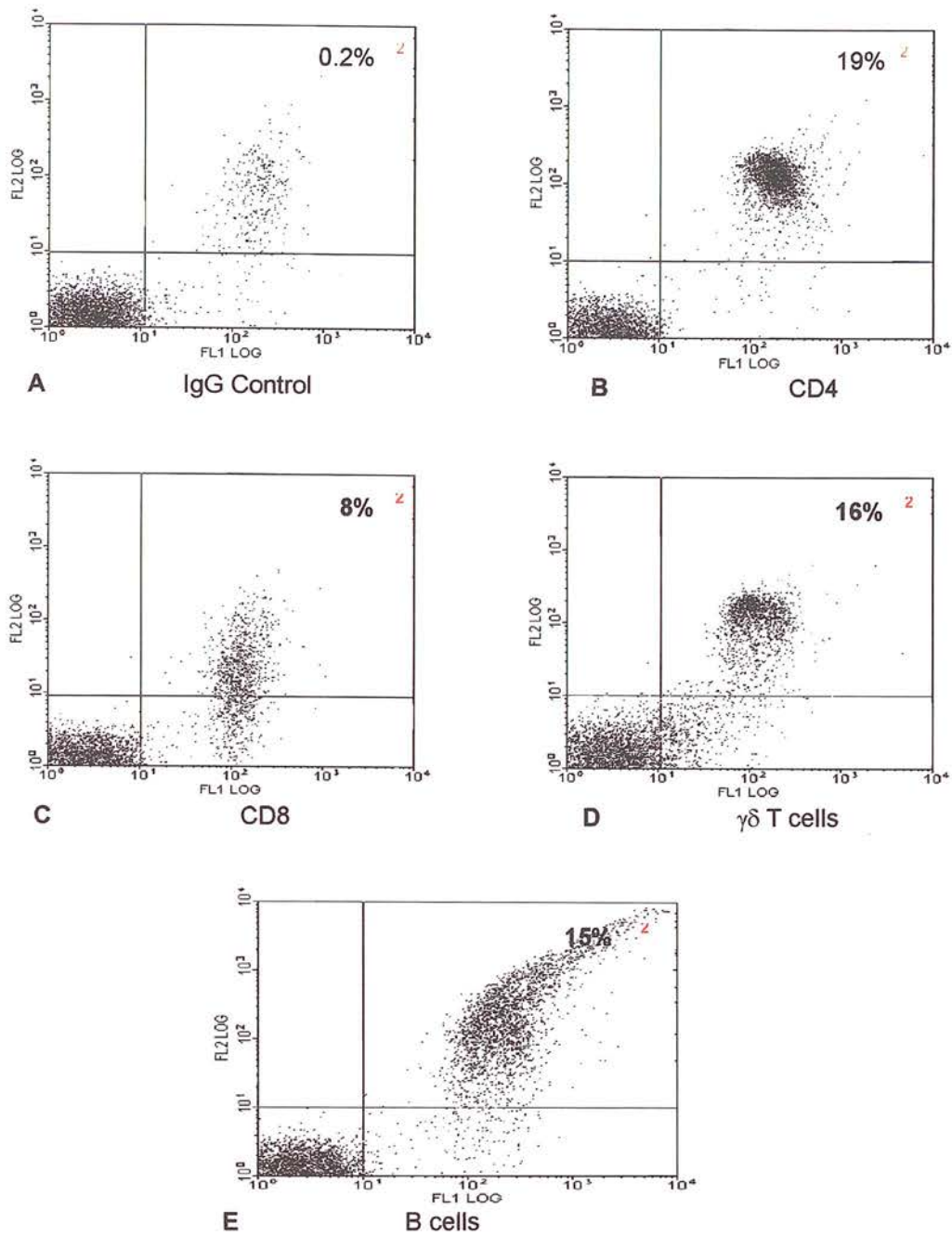


Figure 4.3. Lymphocyte subsets expressing cell surface receptors for NPA. The PBMC purified from the blood of a 2-month-old lamb were labelled with NPA/FITC, and counter-labelled with a panel of monoclonal antibodies/PE (including a mouse IgG control). The percentage lymphocytes/blood (B-E), expressing the receptor for NPA was determined by dual colour flow cytometry. The results are shown as the percentage of double-labelled cells in the peripheral blood, identified in quadrant 2 of each dot plot.

percentage of positively double-labelled cells in the blood detected by the CD2 monoclonal antibody was recorded at 32%. The percentage of positively labelled cells in the blood, detected by the remainder of the monoclonal antibody panel was: CD4 (19%), CD8 (8%), $\gamma\delta$ T cells (16%) and B cells (15%).

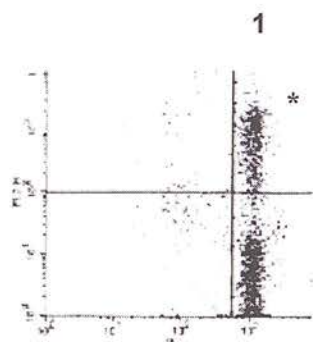
4.2.2 Identification of the PBMC subsets responding to NPA stimulation

4.2.2.1 Methodology

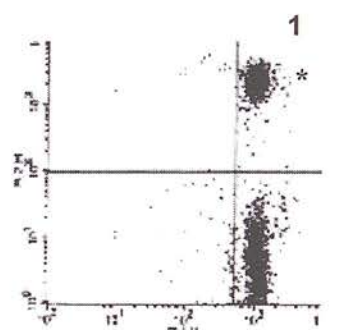
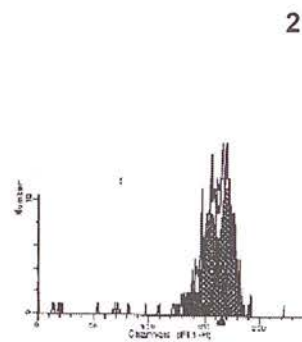
A 20ml blood sample was collected from a two-month-old lamb and the lymphocyte subsets responding to NPA stimulation were identified by carboxyfluorescein diacetate succinimidyl ester (CFSE)/monoclonal antibody selection as detailed in Chapter 2, section 2.8, using a panel of monoclonal antibodies specific for CD2, CD4, CD8, $\gamma\delta$ T cells and B cells.

4.2.2.2 T and B lymphocytes respond to NPA stimulation

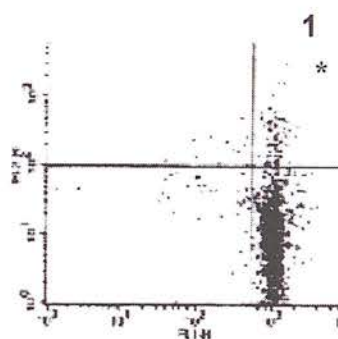
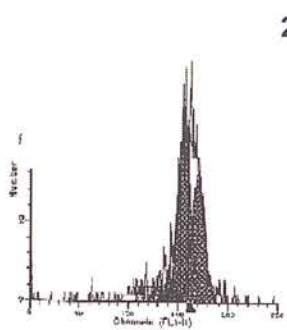
The CFSE results (Figure 4.4 a-e, 1: scatter profile, 2: responder cell histograms, 3: table of percentage cells detected in each generation) revealed that 40.1% of CD2⁺ T cells remained in the parent population, with 40.4% cells detected in generation two having responded to NPA stimulation, and 9% continued to generation three. The CD2 T cell response was further characterised by CD4 and CD8 antibody selection. In response to NPA stimulation, 30.4% CD4⁺ T cells were detected in generation two, 14.1% in generation three. The percentage of CD4⁺ T cells continued to decrease from 4.8% in generation four to <1% from generation seven onwards. The results showed that CD8⁺ T cells did not respond to NPA stimulation with 92.1% retained in the parent generation, and only 5.6% CD8⁺ T cells detected in generation two. The percentages of CD8⁺ T cells in generation three, and subsequent generations were <1%. The parent generation of $\gamma\delta$ T cells contained



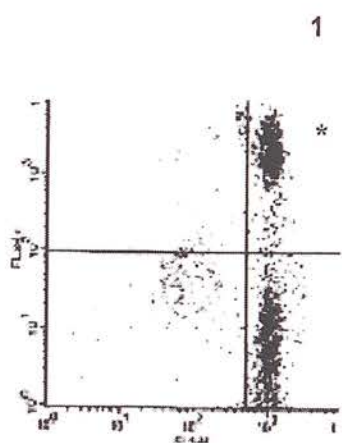
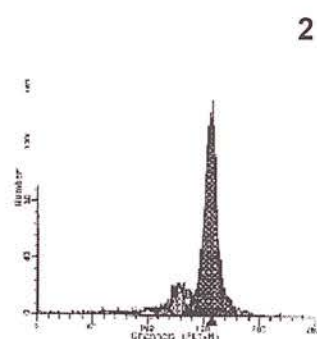
a CD2



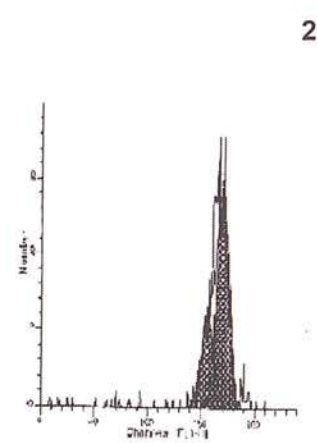
b CD4

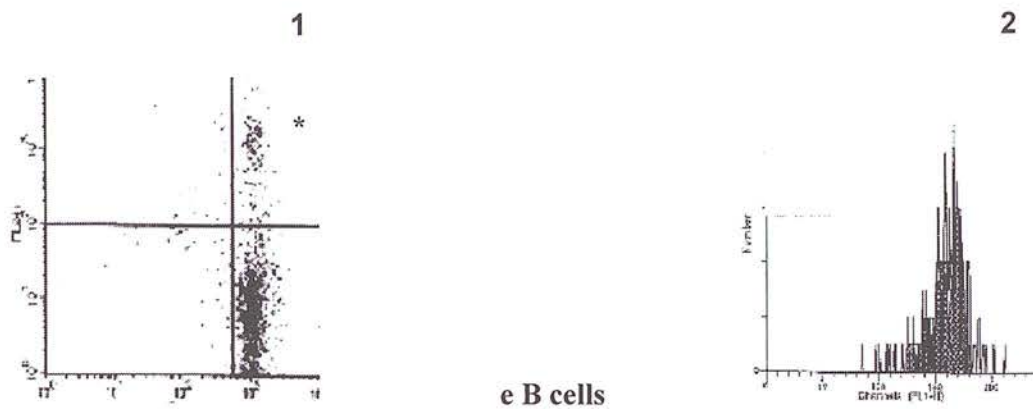


c CD8



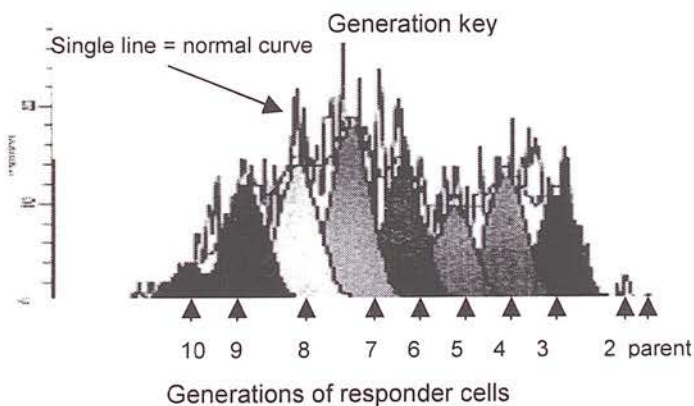
d $\gamma\delta$ T cell





| Table 3 | parent | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|------------------------|--------|------|------|------|-----|-----|-----|-----|-----|-----|
| CD2 | 40.1 | 40.4 | 9.0 | 3.6 | 1.8 | 2.8 | 0.2 | 0.9 | 0.9 | 0.0 |
| CD4 | 46.4 | 30.4 | 14.1 | 4.8 | 1.4 | 1.9 | 0.7 | 0.0 | 0.0 | 0.0 |
| CD8 | 92.1 | 5.6 | 0.8 | 0.3 | 0.0 | 0.0 | 0.6 | 0.4 | 0.0 | 0.0 |
| $\gamma\delta$ T cells | 68.4 | 28.8 | 0.4 | 0.4 | 0.1 | 0.2 | 0.2 | 0.9 | 0.2 | 0.0 |
| B cells | 6.4 | 67.7 | 6.2 | 10.6 | 2.8 | 1.9 | 1.1 | 1.4 | 0.8 | 0.7 |

Figure 4.4 Carboxyfluorescein diacetate succinimidyl ester (CFSE) /monoclonal antibody detection of lymphocyte subsets responding to NPA stimulation. The PBMC were purified from the blood of a 2-month-old lamb. Prior to stimulation with NPA, CFSE was incorporated into the cells. The cells were collected, post-NPA stimulation and phenotyped with a panel of monoclonal antibodies specific for CD2, CD4, CD8, $\gamma\delta$ T cells and B cells. The results are shown for each lymphocyte subset (A - E) as 1: scatter profile with responder cells detected in * top right quadrant, 2: histogram of generations proliferating in response to NPA and 3: table of percentage cells detected in each generation.



Software modelling was used to generate populations of dividing cells by the use of computer fitting to determine the series of curves which accommodate the experimental data. The proportion of cells in each generation (cell division) was determined by the relative area under each normal curve.

68.4%, with 28.8% detected in generation two and from generation three onwards <1% $\gamma\delta$ T cells were detected. The results provided evidence that B cells proliferated in response to NPA stimulation with 67.7% detected in generation two. Although only 6.2% B cells were found in generation three this percentage increased to 10.6% in generation four. The percentage of B cells in subsequent generations decreased from 2.8% in generation five to <2% in generations six-eight, and <1% in generations nine-ten.

To summarise the results: evidence of lymphoproliferation was demonstrated for both T and B lymphocytes. Further characterisation of the T cell population identified that the $CD4^+$ subset were the most predominant responder cells to NPA, and by comparison $\gamma\delta$ T cells showed a limited response. No evidence of $CD8^+$ T cell responses were detected. It would appear from the results that B cells responded also, with the majority of B cells progressing to generation two. The percentage of B cells increased from 6.2% in generation three to 10.6% in generation four, signifying a proliferative response. This observation may indicate a lymphoproliferative response by a subpopulation of B cells that is T cell directed.

4.3 Lymphoproliferation to NPA stimulation is adversely affected by the depletion of adherent cells from the PBMC

The PBMC from four lambs were purified (Chapter 2, section 2.2) and the lymphoproliferative responses to NPA stimulation in the entire-PBMC, adherent-cell-depleted-PBMC and the reconstituted PBMC were compared (Chapter 2, section 2.5.2). The data were analysed and group results compiled (Chapter 2, section 2.9). A statistically significantly reduced response to NPA stimulation was demonstrated

in the adherent-cell-depleted-PBMC ($258 \text{ cpm} \pm 54$) compared with the levels of response identified for the entire-PBMC (2111 ± 373 ($p = 0.003$)) and reconstituted-PBMC (1687 ± 318 ($p = 0.004$)), as shown in Figure 4.5.

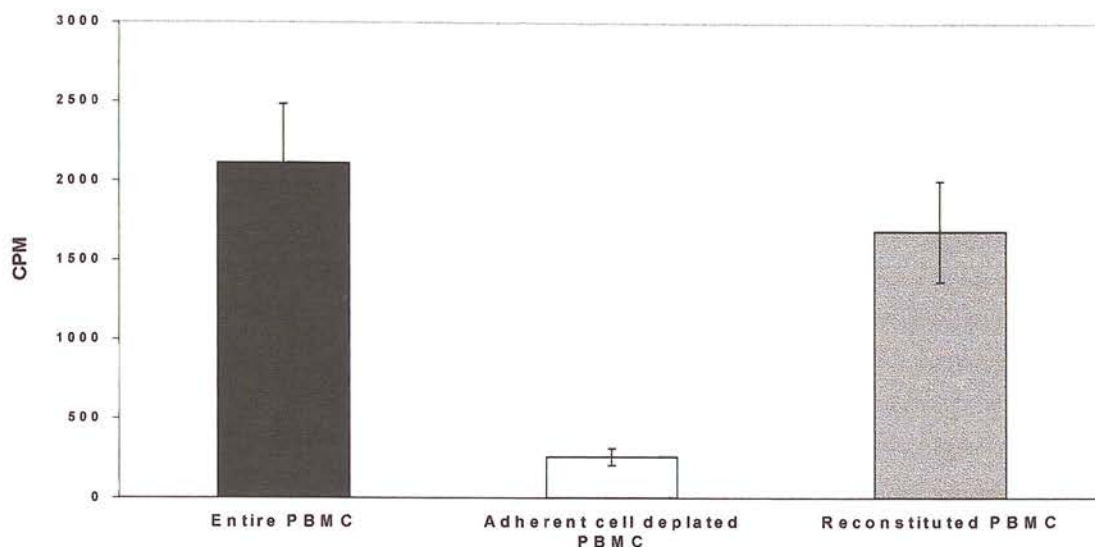


Figure 4.5 The influence of antigen presenting, adherent cells in the mitogenicity of NPA for sheep lymphocytes. The lymphoproliferative responses to NPA stimulation of the entire-PBMC, adherent-cell-depleted-PBMC and reconstituted-PBMC (replacement of adherent cells) were measured in blood samples collected from 4 lambs. Results are shown as counts per minute (cpm) \pm SEM, determined by ^3H -thymidine uptake with the unstimulated PBMC (135 cpm) subtracted. The results show that the lymphoproliferative response to NPA stimulation of the adherent-cell-depleted-PBMC is significantly reduced compared with the entire PBMC ($p=0.003$), and the reconstituted PBMC ($p=0.004$).

4.4 NPA mitogenicity during JSRV infection

4.4.1 Lymphoproliferation to NPA stimulation in JSRV-infected and uninfected lambs

The lymphoproliferative responses (Chapter 2, section 2.5) to NPA stimulation were measured in the purified PBMC (Chapter 2, section 2.2) from six experimentally JSRV-infected lambs aged 2, 8, 12 and 16 weeks, and compared with

the responses detected in four, age-matched control lambs. No significant difference in the level of response to NPA stimulation was identified at any time between the two groups: 2 weeks (JSRV-infected 1665 cpm \pm 688, controls 2211 cpm \pm 909), 8 weeks (JSRV-infected 4493 cpm \pm 2264, controls 5404 cpm \pm 2883), 12 weeks (JSRV-infected 1440 cpm \pm 1038, controls 2057 cpm \pm 1467) and 16 weeks (JSRV-infected 2028 cpm \pm 767, controls 1278 cpm \pm 641) (Figure 4.6). Therefore, it is evident from this study that the lymphoproliferative response to NPA mitogenicity is not altered during JSRV infection.

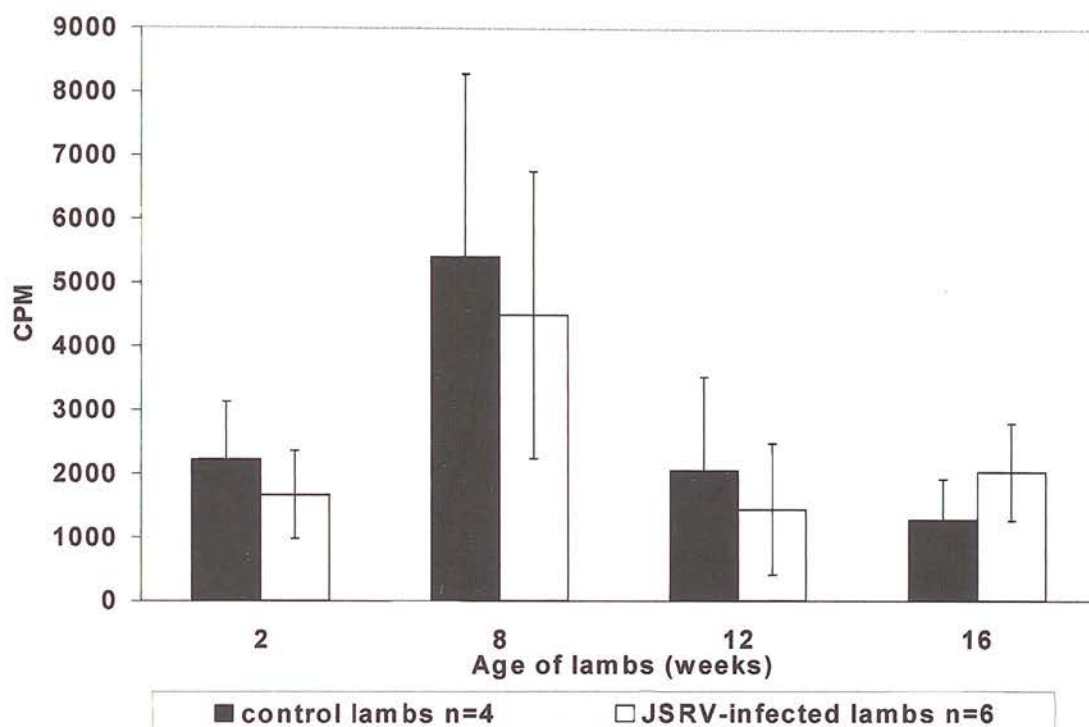


Figure 4.6 Lymphoproliferation to NPA stimulation in JSRV-infected and control lambs. The lymphoproliferative responses of PBMC from 6 JSRV-infected and 4 control lambs was measured and compared at 2, 8, 12 and 16 weeks post-infection. The results are shown as counts per minute (CPM) \pm SEM, determined by ^3H -thymidine uptake. The unstimulated PBMC (2 weeks 130 : 275, 8 weeks 167 : 171, 12 weeks 128 : 160 and 16 weeks 135 : 193 cpm, control and JSRV-infected lambs respectively) were subtracted. No significant difference in the level of response was detected between the infected and control groups, at any time point tested.

4.4.2 Lymphocyte profile of JSRV infected and control lambs pre and post-NPA-stimulation

4.4.2.1 Methodology

The PBMC from three experimentally JSRV-infected and three control lambs were purified (Chapter 2, section 2.1) and adjusted to a final concentration of 10^6 PBMC/ml as detailed in Chapter 2, section 2.2. Aliquots of 5×10^5 PBMC were phenotyped with monoclonal antibodies recognising CD2⁺ T and B lymphocytes, and the results determined by flow cytometry according to the protocols detailed in Chapter 2, section 2.3. The remaining cells were stimulated with NPA (Chapter 2, section 2.5.1). Post-stimulation, the cells were collected by aspiration and centrifuged at 300g for 5 minutes. The supernatant was decanted and the cell pellet was re-suspended in 1ml PBS. The PBMC were counted (Chapter 2, section 2.2.2) and phenotyped following the protocol for the un-stimulated PBMC detailed above.

The numbers of immunolabelled T and B lymphocytes/ml blood, pre-and-post-stimulation, were calculated for each animal (Chapter 2, section 2.9).

4.4.2.2 The presence of JSRV alters the lymphocyte profile post-NPA stimulation

The phenotypic profile of CD2 T cells and B cells from control and JSRV-infected lambs, pre-and post-NPA stimulation, are shown in Figure 4.7.

CD2 T cells: The difference in the mean of the actual number of immunolabelled T cells/ml blood from the control group pre-stimulation ($1.3 \times 10^6 \pm 6.7 \times 10^4$), compared with post-stimulation ($1.9 \times 10^4 \pm 1.1 \times 10^4$) was found to be highly significant ($p=0.00005$). In contrast, no significant difference was detected in the JSRV-infected group (pre-stimulation: $8.1 \times 10^5 \pm 3.4 \times 10^5$, post-stimulation $2.3 \times 10^5 \pm 5.5 \times 10^4$).

B cells: The difference in the mean of the actual numbers of immunolabelled B cells/ml blood from the control group pre-stimulation ($1.1 \times 10^6 \pm 2.1 \times 10^5$), compared with post-stimulation ($2.5 \times 10^4 \pm 9.0 \times 10^3$) was found to be statistically significant ($p=0.01$). No significant difference was detected in the JSRV-infected group (pre-stimulation: $4.0 \times 10^5 \pm 1.3 \times 10^5$, post-stimulation $4.1 \times 10^5 \pm 5.7 \times 10^4$).

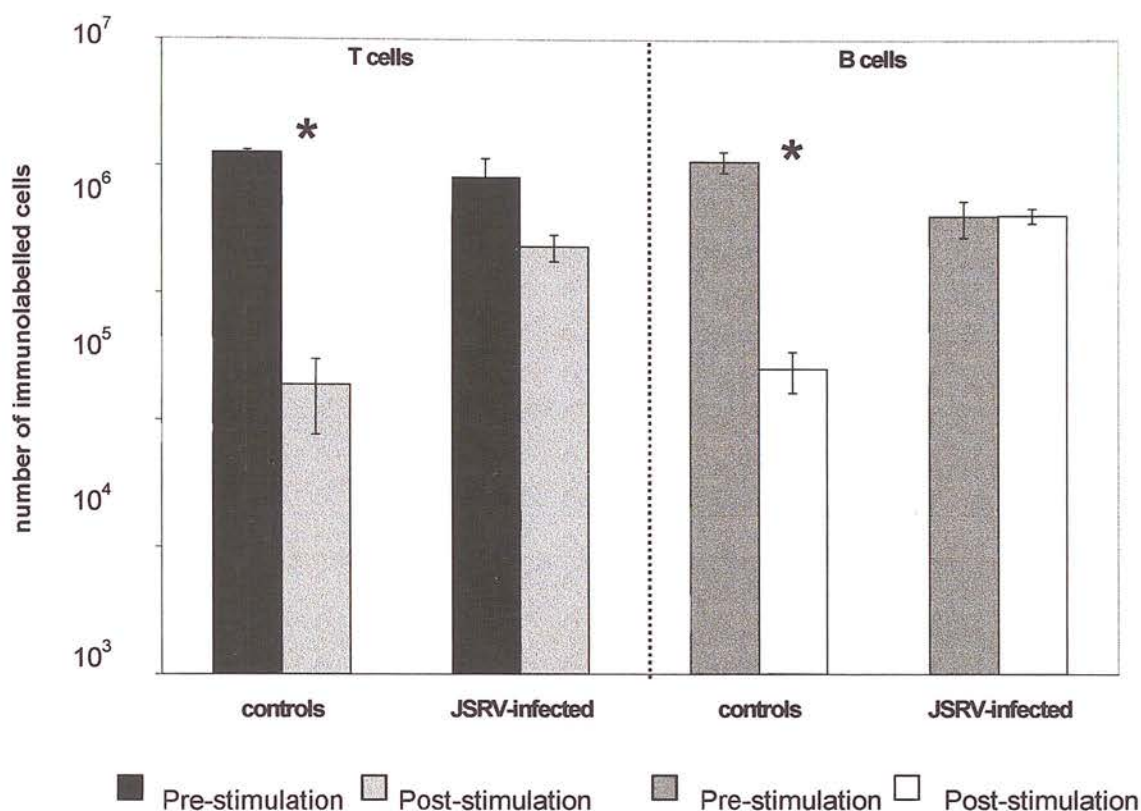


Figure 4.7 T and B cell phenotyping of JSRV-infected and control lambs, pre and post NPA stimulation. The frequency of T and B cells in the blood of 3 JSRV-infected and 3 control lambs were compared, before and after stimulation with NPA, by immunolabelling with monoclonal antibodies and the results determined by flow cytometry. The results are shown as the mean number of immunolabelled cells/ml blood, \pm SEM. In the control group, post-stimulation, statistically significant decreases ($*p=0.00005$ and $*p=0.01$) in the number of T and B cells were detected compared to pre-stimulation. No significant difference (pre and post NPA stimulation) was detected in the numbers of T and B cells from the JSRV-infected lambs.

4.5 Inhibition of NPA mitogenicity by the addition of mannose

4.5.1 Optimisation of the mannose concentration required to induce maximum inhibition of NPA mitogenicity.

4.5.1.1. Methodology

To test the hypothesis that the mitogenicity of NPA could be inhibited by the addition of exogenous mannose, prior to PBMC stimulation, it was necessary to determine the optimum mannose concentration required. Various concentrations of mannose were added to 100 μ g NPA/ml for 24 hours at 4⁰C. The PBMC were purified from the blood of a three-month-old lamb (Chapter 2, section 2.2.1) and adjusted to a final concentration of 10⁶ PBMC/ml (Chapter 2, section 2.2.2). Lymphoproliferation assays were performed as described in Chapter 2, section 2.5.1 with 100 μ g/ml NPA, NPA/mannose concentration series and PBMC control (no lectin added). The data were analysed as detailed in Chapter 2, section 2.9.

4.5.1.2 Mannose inhibits NPA mitogenicity in a control lamb

As shown in Figure 4.8, cpm in response to stimulation with NPA alone was calculated at 1545 cpm. The lymphoproliferative response was decreased when the PBMC were stimulated with NPA/mannose (1mM: 181cpm, 10mM: 300cpm, 100mM: 17cpm, 200mM: 206cpm, 400mM: 141cpm), after the unstimulated PBMC 73 cpm was subtracted. Although the mitogenicity of NPA was suppressed at all the concentrations tested, the optimum inhibitory concentration of mannose was found to be 100mM/100 μ g NPA, which resulted in almost complete inhibition.

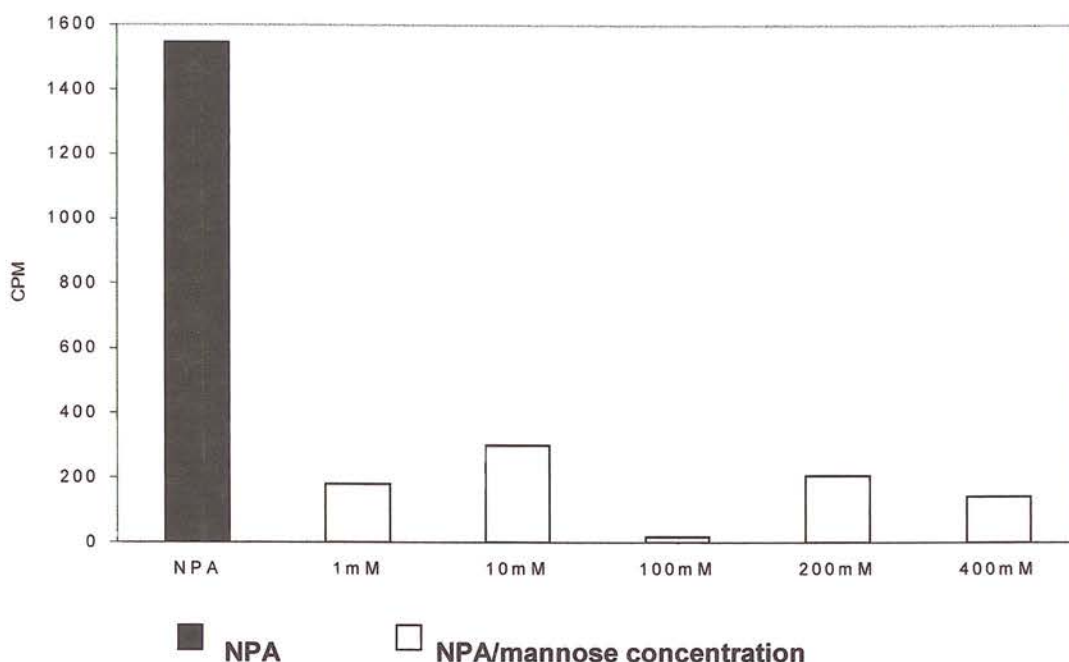


Figure 4.8 Optimum mannose concentration required to inhibit NPA mitogenicity, in an uninfected lamb. The PBMC purified from the blood of a 3-month-old lamb were stimulated with NPA alone and NPA with the addition of 1mM, 10mM, 100mM, 200mM and 400mM mannose. The results are shown as counts per minute (cpm), determined by ^3H -thymidine uptake. The inhibitory effect of mannose was demonstrated at all concentrations tested, with the optimum concentration 100mM which resulted in almost complete inhibition of NPA mitogenicity.

4.5.1.3 Mannose only partially inhibits NPA mitogenicity in a JSRV-infected lamb

The inhibitory effect of mannose was measured in the PBMC from a JSRV infected lamb to determine if the optimum mannose concentration required to inhibit NPA in the control lamb was consistent for an experimentally JSRV-infected lamb. The optimisation assay detailed above was repeated with the PBMC purified from the blood of an age matched, experimentally JSRV-infected lamb. The results showed that stimulation of the PBMC with NPA alone generated 2438 cpm, after the

unstimulated PBMC 142 cpm was subtracted. In comparison, the addition of 1mM mannose did not inhibit NPA mitogenicity (2484 cpm), and the addition of 10mM mannose reduced the lymphoproliferative response by only 27%. At the optimum concentration (100mM) identified for the control lamb, 54% lymphoproliferation was demonstrated with the JSRV-infected lamb. At higher concentrations (200mM and 400mM) mannose was not inhibitory. Therefore, it was determined from the resulting standard curve that the addition of 100mM mannose did result in the highest level of inhibition, albeit only partial, and complete inhibition was not demonstrated at any concentration tested (Figure 4.9).

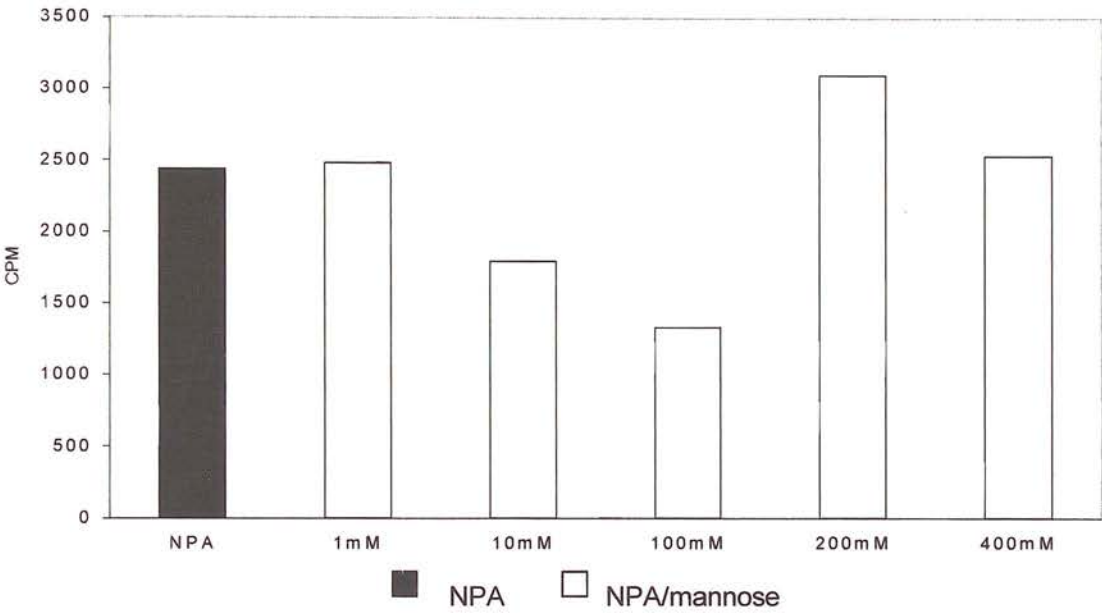


Figure 4.9 Optimum mannose concentration required to inhibit NPA mitogenicity in a JSRV-infected lamb. The PBMC purified from the blood of a 3-month-old, experimentally JSRV-infected lamb was stimulated with NPA alone and NPA with the addition of 1mM, 10mM, 100mM, 200mM and 400mM mannose. The results are shown as counts per minute (cpm), determined by ³H-thymidine uptake. The addition of 10mM and 100mM only partially inhibited the mitogenicity of NPA. At all other concentrations tested, the lymphoproliferative response was not inhibited. The optimum inhibitory concentration of mannose was found to be 100mM, with only partial inhibition detected.

4.5.2 Mannose inhibition of NPA mitogenicity during experimental JSRV infection.

4.5.2.1 Methodology

The investigation of the inhibitory effect of mannose on NPA mitogenicity identified during the optimisation assays, was extended to include experimentally JSRV-infected and control lambs aged 1, 2, 4, 8, 10, 16 and 18 weeks. At each time point the PBMC were purified (Chapter 2, section 2.2) from the blood of three JSRV-infected lambs at 1, 2, 4 and 8 weeks and two JSRV-infected lambs at 10, 16 and 18 weeks. The PBMC purified from the blood of two control lambs were used throughout. At each time point the PBMC were stimulated with NPA alone, NPA/mannose and PBMC controls (no lectin added). Lymphoproliferation was performed as described in Chapter 2, section 2.5. The data were analysed and the group results compiled as detailed in Chapter 2, section 2.9.

4.5.2.2 Mannose inhibition of NPA mitogenicity is age-related in uninfected lambs and during JSRV infection

Control lambs (Figure 4.10a) The addition of mannose did not inhibit the lymphoproliferative response to NPA stimulation in one to four week old control lambs. Moreover the responses detected at two weeks (NPA: 3756cpm \pm 216, NPA/mannose: 9050cpm \pm 1949) and four weeks (NPA:10172cpm \pm 497, NPA/mannose: 12248cpm \pm 679) were found to be enhanced by the presence of mannose in comparison with those detected with NPA alone, although this increase was not statistically significant ($p=0.1$). At eight weeks a decrease in the lymphoproliferative response was observed in the NPA/mannose samples (NPA/mannose 1245cpm \pm 104), compared with the response to NPA alone (2097cpm \pm 930). From ten weeks onwards no lymphoproliferative response was

detected in the NPA/mannose samples, demonstrating complete inhibition of NPA mitogenicity.

JSRV-infected lambs (Figure 4.10b) The responses detected to NPA stimulation in lambs aged one to eight weeks (1 week: $1953\text{cpm} \pm 1049$, 2 weeks: $2868\text{cpm} \pm 1258$, 4 weeks: $5308\text{cpm} \pm 640$ and 8 weeks: $4341\text{cpm} \pm 345$) were not inhibited by the addition of mannose (1 week: $4031\text{cpm} \pm 1881$, 2 weeks: $3880\text{cpm} \pm 1034$, 4 weeks: $6783\text{cpm} \pm 872$ and 8 weeks: $8269\text{cpm} \pm 673$). The enhanced lymphoproliferation detected in the NPA/mannose samples at two and four weeks, compared with the response identified to NPA stimulation, was not significant. However, the increased lymphoproliferation detected in the NPA/mannose sample compared with the response to NPA alone at *8 weeks was statistically significant ($p=0.007$). At ten weeks the lymphoproliferative response to NPA and NPA/mannose stimulation was comparable ($4640\text{cpm} \pm 1304$, $3923\text{cpm} \pm 1354$, respectively). At sixteen weeks (NPA: $4048\text{cpm} \pm 666$, NPA/mannose $1589\text{cpm} \pm 445$) and eighteen weeks (NPA: $6668\text{cpm} \pm 2788$, NPA/mannose $2744\text{cpm} \pm 2000$) only partial inhibition of NPA mitogenicity was detected. Therefore no evidence of complete inhibition was demonstrated at any time point, in the JSRV-infected lambs.

4.6 NPA mitogenicity in human subjects

To investigate the human lymphoproliferative response to NPA stimulation, the PBMC from the blood of adult volunteers aged 24 -56 years were compared with the response measured in umbilical cord blood. Unfortunately it is not possible to readily obtain blood samples from children, but ethical permission was granted for preliminary studies to be undertaken with mononuclear cells purified from umbilical cord blood.

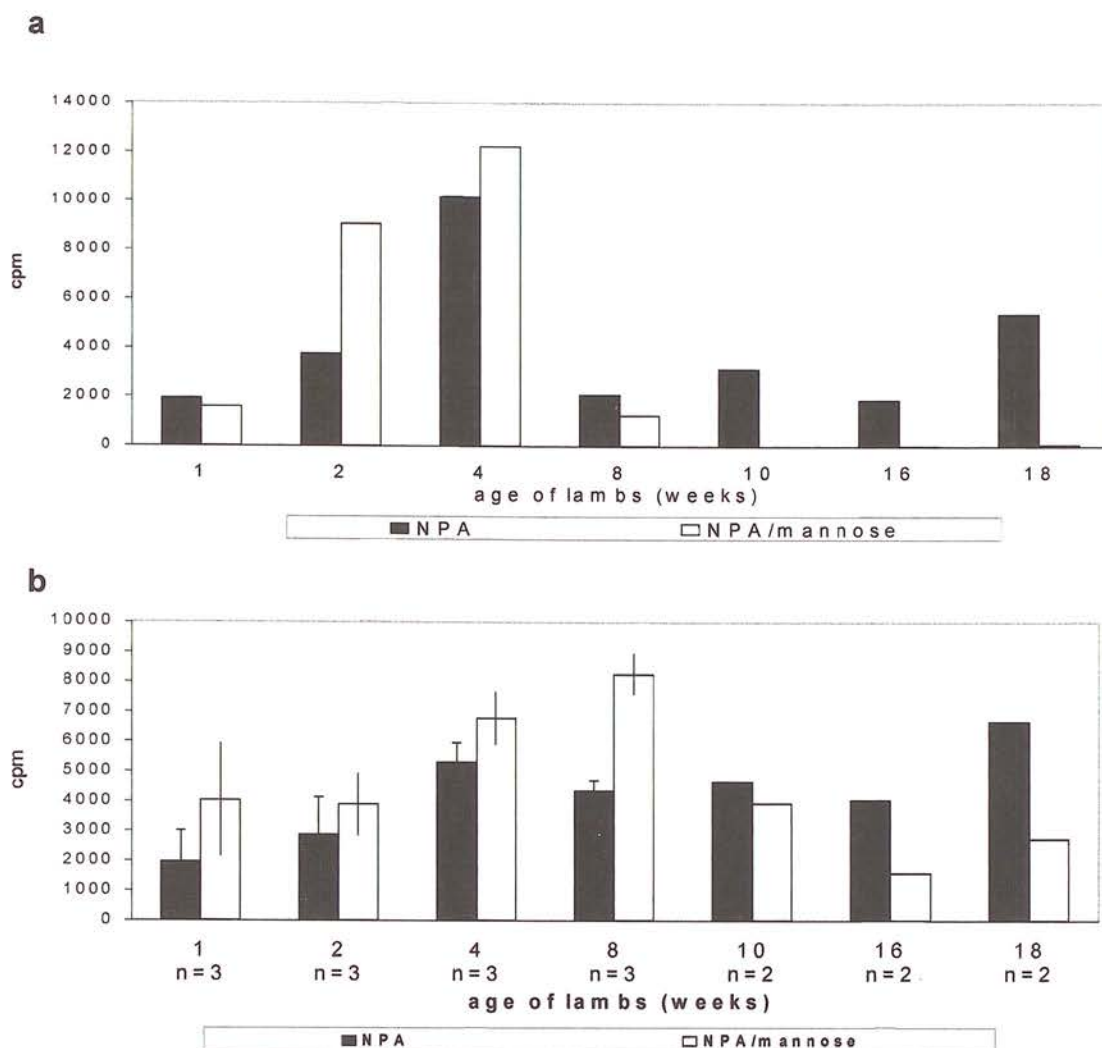


Figure 4.10 Mannose inhibition of NPA mitogenicity during JSRV infection. The lymphoproliferative response to stimulation with NPA alone and NPA/mannose was tested in JSRV-infected and control lambs, at 1, 2, 4, 8, 10, 16 and 18 weeks old. At each time point, the PBMC were purified from the blood of 2 control lambs and 3 experimentally JSRV-infected lambs at 1, 2, 4 and 8 weeks, 2 JSRV-infected lambs at 10, 16 and 18 weeks. The results are shown as counts per minute (cpm) \pm SEM, determined by ^3H -thymidine uptake with the unstimulated PBMC cpm (1 week 128 : 95, 2 weeks 194 : 103, 4 weeks 388 : 161, 8 weeks 219 : 114, 10 weeks 274 : 145, 16 weeks 330 : 227 and 18 weeks 192 : 139 cpm, control and JSRV-infected lambs respectively. Figure 4.10a: The lymphoproliferative response to NPA and NPA/mannose detected in control lambs aged 1-4 weeks revealed that the presence of mannose had no inhibitory effect on NPA mitogenicity. From 10 weeks onwards, the addition of mannose resulted in complete inhibition of NPA mitogenicity. Figure 4.10b: The lymphoproliferative response to NPA and NPA/mannose detected in JSRV-infected lambs in lambs aged 1-8 weeks, revealed that the presence of mannose did not inhibit NPA mitogenicity. At 8 weeks a significant increase ($p=0.007$) in the response to NPA/mannose stimulation, compared with NPA alone was demonstrated. Only partial inhibition of NPA mitogenicity was detected from 10 weeks onwards.

4.6.1 Methodology

4.6.1.1 Blood samples

In total, twelve human blood samples were collected, from five volunteer adults, ages ranging from 24-56 years, and seven umbilical cord bloods obtained during routine caesarean sections (kindly collected by Dr. Rhona Hughes, Edinburgh Royal Infirmary, with informed consent). All blood samples were collected into 10units/ml preservative free heparin. The volume of umbilical cord blood ranged from 2.5 – 5ml, with 5ml adult peripheral blood collected

4.6.1.2 Mononuclear cell purification

The umbilical cord mononuclear cells (UCMC) and the adult PBMC were purified within four hours of collection by layering the whole blood over 5ml Lymphoprep™ (Nycomed Pharma AS, Oslo, Norway), followed by centrifugation at 300g for 15 minutes. The interface cells were recovered and re-suspended in 10ml RPMI-1640 containing antibiotics (Chapter 2, section 2.2), 10% autologous plasma and 10units heparin/ml. The samples were centrifuged at 240g for 10 minutes and the supernatant decanted. The cell pellet was then washed twice by centrifugation at 240g for 5 minutes and re-suspended in 5ml RPMI-1640 medium (no added heparin). After a final wash the supernatant was decanted and the cells re-suspended in 5ml RPMI-1640 with 10% autologous plasma. The cells were adjusted to a final concentration of 10^6 cells/ml (Chapter 2, section 2.2.2).

4.6.1.3 proliferation assays

The lymphoproliferative response of the UCMC and PBMC to PHA and NPA stimulation was determined as described in Chapter 2, section 2.5. The data was analysed and group results compiled (Chapter 2, section 2.9).

4.6.2 Optimisation of NPA concentration for human lymphocytes

The mononuclear cells purified from two umbilical cords and an adult blood were stimulated with various concentrations of NPA to determine the concentration of lectin required to produce maximum lymphoproliferative response. This was found to be 100µg/ml for UCMC and adult PBMC, (Figure 4.11).

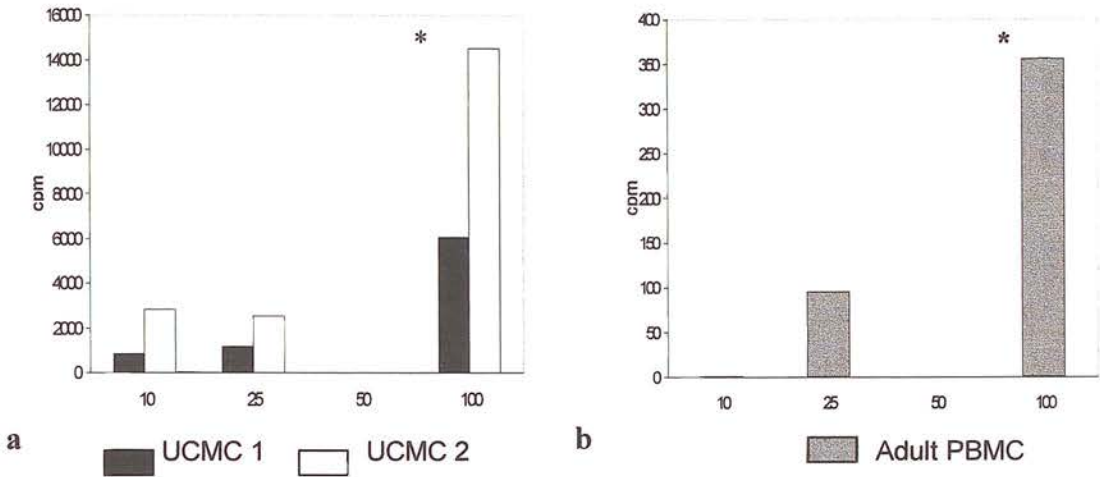


Figure 4.11 NPA concentrations tested to induce maximum lymphoproliferation in humans. The results are shown as counts per minute (cpm), determined by ³H-thymidine uptake with the unstimulated PBMC (UCMC1: 204. UCMC 2: 367 and adult PBMC: 178 cpm) subtracted. Figure 4.11a: The UCMC from two cord bloods were stimulated with 10, 25 and 100µg/ml NPA. Figure 4.11b: The PBMC from adult blood was stimulated with 10, 25 and 100µg/ml NPA. The maximum lymphoproliferative response to NPA stimulation was detected *100ug/ml for UCMC and PBMC.

4.6.3 NPA mitogenicity for human lymphocytes is age-related

To identify if NPA mitogenicity was age-related in humans, the UCMC from seven cord bloods and five adult PBMC were purified as detailed in section 4.6.1.2 and stimulated with NPA and PHA.

As shown in Figure 4.12 PHA was strongly mitogenic for lymphocytes of both human age groups (UCMC mean cpm 65058 ± 12108 and adult PBMC 76161 ± 13770) and there was no statistically significant difference in these responses. In

contrast, the lymphoproliferative response to NPA stimulation was significantly higher ($p=0.0004$) with UCMC (mean cpm 11322 ± 1549) compared to adult PBMC (mean cpm 1580 ± 413) (Figure 4.12.).

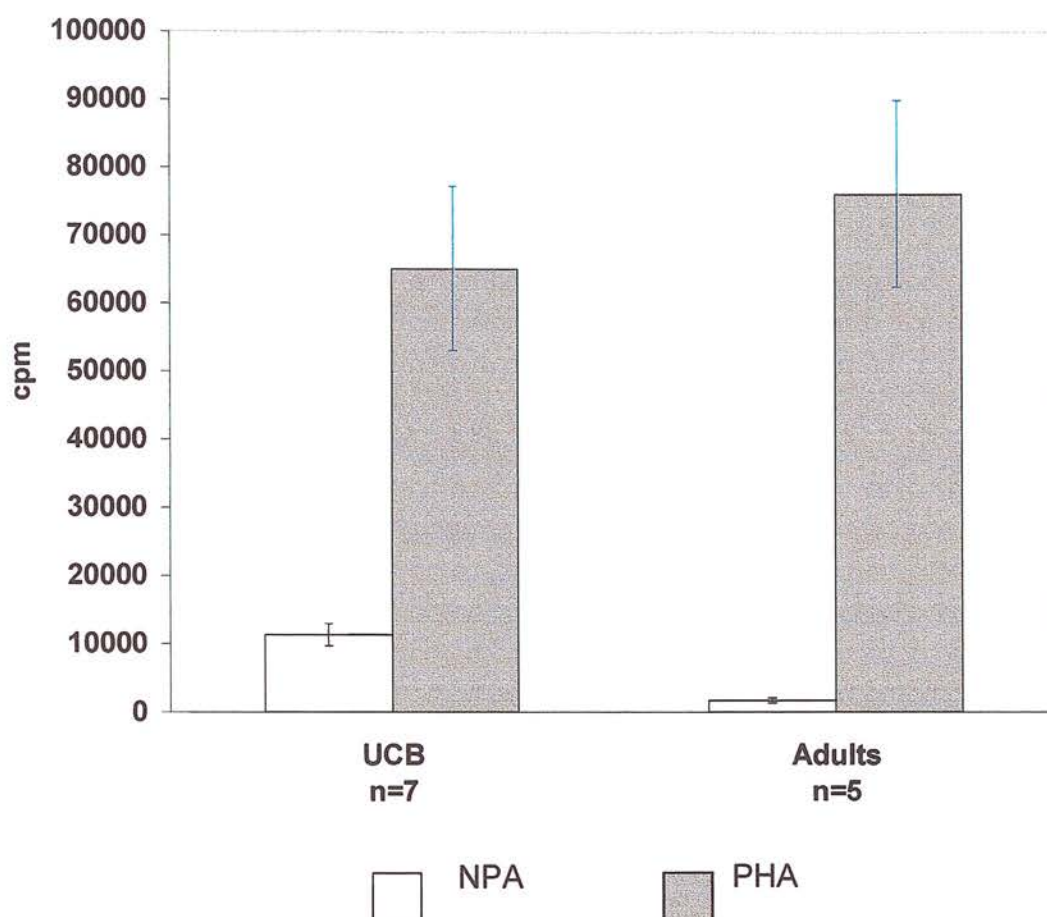


Figure 4.12 Human lymphoproliferative response to PHA and NPA stimulation. The mononuclear cells, purified from 7 umbilical cord bloods (UCMC) and the peripheral blood mononuclear cells (adult PBMC) from 5 adults (aged 24-56 years) were stimulated with PHA and NPA. The results are shown as counts per minute (cpm) \pm SEM, determined by ^3H -thymidine uptake with the unstimulated PBMC (UCMC 197, Adult PBMC 157 cpm) subtracted. Comparison between the two groups was not statistically significant for PHA stimulation, but highly significant ($p=0.0004$) for NPA.

4.7 Discussion

The investigation into the ovine lymphoproliferative response to stimulation by the monocotyledonous *Narcissus pseudonarcissus* lectin (NPA) has identified that mitogenicity for sheep lymphocytes is inversely correlated to the increasing age of the animals. Lymphoproliferation in response to NPA stimulation was demonstrated in lambs as young as four days old and effective mitogenicity was still evident when the animals were at least four months of age. No evidence to support NPA mitogenicity in adult sheep was detected (section 4.1.2).

It was established that all the lymphocyte subsets tested in the PBMC have a cell-surface receptor for NPA (section 4.2.1), although the percentages of double-labelled cells detected appeared very low in comparison with subset percentages detected in other studies. This was particularly true for $\gamma\delta$ T cells, the predominant T cell subset in young lambs (Hein & McKay 1991). It has been established that in sheep, the percentage range of peripheral blood lymphocytes varies considerably between individual animals, for example CD4 (8-22%), CD8 (4-20%), $\gamma\delta$ T cells (22-68%) and B cells (11-50%) have been demonstrated (McKay 1986, McKay *et al* 1988, Hein & McKay 1991, Smith *et al* 1994). A study by Bachh *et al* (1995) demonstrated ovine reactivity to monoclonal antibodies in lambs aged three to four months and identified the percentage distribution of positively immunolabelled lymphocyte subsets in the peripheral blood as CD2 (30.6%), CD4 (16.8%), CD8 (8.8%), $\gamma\delta$ T cells (15.5%) and B cells (19.7%), giving a CD4 : CD8 ratio 1.81:1. The results of the Bacch *et al* study corresponded closely to the previous findings of MacKay & Hein (1987) and Ellis *et al* (1991). In comparison, the smaller percentage of B cells identified (15%) and the higher CD4 : CD8 ratio of 2.3:1 detected during

this study may have resulted because the PBMC were purified from a 2 month old lamb and the lymphocyte distribution would not therefore correspond exactly to the results obtained in the aforementioned studies. Alternatively the low response might be due to the presence of NPA, if binding to the cell surface receptor resulted in blocking attachment of the monoclonal antibody to the cells. To test this hypothesis the assay could be repeated, testing monoclonal antibodies recognising different epitopes and performing the immunolabelling prior to incubation with the FITC conjugated lectin.

In this study lymphoproliferation was demonstrated for T, and possibly B lymphocytes also (section 4.2.2). Further characterization of the T cell population identified that the CD4⁺ subset were the most predominant responder cell to NPA, and $\gamma\delta$ T cells showed a limited response, but no evidence of CD8⁺ T cell responses were detected. If binding of the lectin to the cell does adversely affect subsequent immunolabelling this may account for the very low proliferative response detected.

It was revealed that the level of NPA mitogenicity was influenced by the presence of antigen-presenting-adherent cells (section 4.3), demonstrating that accessory cells are required for the mitogenicity of this lectin, a finding in sheep, which correlates with NPA studies performed with human lymphocytes by Kilpatrick *et al* (1990). Habu and Raff first described the influence of accessory cells on the mitogenicity of several lectins in 1977. Subsequent studies have demonstrated that the presence of antigen-presenting-adherent cells, such as monocytes, dendritic cells, and/or non-adherent B cells are required for the mitogenicity of several lectins. For some lectins the physical presence of antigen presenting cells is all that is necessary to enhance mitogenicity, by localising the lectin and promoting cross-linking of the

receptors present on the lymphocyte surface (Gallagher *et al* 1986). More subtle factors provided by antigen presenting cells control the level of mitogenicity of other lectins. For example, wheat germ agglutinin (WGA), (another monocotyledonous lectin, but not mannose specific) derived from the grass family, depends strongly on the soluble mediators produced by accessory cells, such as IL-1 (Kilpatrick *et al* 1988). WGA mitogenicity for antigen-presenting-cell-depleted-PBMC from human blood has only been demonstrated with the addition of exogenous IL-2, suggesting therefore, that WGA, although binding to the lymphocytes directly, and consequently inducing IL-2R expression does not induce IL-2 production directly. It is the soluble mediators produced by the monocytes that are required to stimulate the production of this monokine, and therefore provide the essential components necessary for WGA mitogenicity (Kilpatrick *et al* 1988). Not all lectins are so dependent on accessory cells for mitogenicity, as demonstrated with PHA and poke weed mitogen (PWM) during this project (Chapter 3, section 3.4), and by several others (reviewed by Kilpatrick 1999).

The lymphoproliferative response to NPA stimulation was not adversely affected during experimental JSRV-infection, with the level of stimulation comparable in JSRV-infected and control lambs (section 4.4.1). However, the T and B lymphocyte profiles identified post-NPA stimulation in the blood of JSRV-infected lambs were altered, in comparison with the control animals (section 4.4.2). In the PBMC from the control lambs the numbers of immunolabelled cells, post-stimulation, were found to be significantly reduced (T cells $p=0.00005$, B cells $p=0.01$), in comparison to the numbers detected pre-stimulation. These results were not as expected. It was envisaged, that as lymphoproliferation had occurred, a greater

number of positively immunolabelled lymphocytes (post-stimulation) would be detected. The reason for this observation remains unclear but could again suggest that the receptor for NPA in control animals blocks attachment of the monoclonal antibodies, decreasing the number of immunolabelled cells detected, or that proliferation with NPA may result in downregulating the CD2 and B cell ligands. By comparison, no significant difference in the numbers of T and B lymphocytes were detected before and after NPA stimulation, with the JSRV-infected lambs. This observation could indicate that during JSRV infection, proliferation with NPA results in the death of an inhibiting cell or that NPA utilises a receptor that does not interfere with monoclonal antibody binding. Perhaps therefore, NPA mitogenicity could be induced *via* different mechanisms in control and JSRV-infected lambs.

Further evidence to support the observation that the presence of JSRV has a direct effect on the lymphocytes, and that NPA mitogenicity may be induced through a different pathway during experimental infection, was demonstrated by the altered cellular response in the JSRV-infected lambs to mannose inhibition of NPA mitogenicity (section 4.5). If lymphoproliferation occurs *via* the mannose pathway, it was hypothesised that the addition of exogenous mannose to NPA, prior to incubation with the PBMC, would result in inhibiting mitogenicity. The addition of exogenous mannose did result, as hypothesised, in complete inhibition in uninfected lambs aged ten to eighteen weeks. In contrast, mannose only partially inhibited NPA-induced proliferation in age-matched JSRV-infected lambs. It was further demonstrated that the addition of mannose was not inhibitory to NPA mitogenicity in JSRV-infected or control lambs, aged one to eight weeks old, indicating that, in young lambs, NPA mitogenicity was not *via* the mannose pathway. In the JSRV-

infected and control lambs, at ten weeks old, the trend towards inhibition was observed, culminating in the complete inhibition of NPA mitogenicity in uninfected lambs. Thus the inhibitory affect of mannose was age-related. In contrast, complete inhibition was not detected at any time point with the age-matched, JSRV-infected lambs. This may suggest that lymphoproliferation to NPA stimulation was not induced *via* the mannose pathway in JSRV-infected lambs. If a different mechanism for mitogenesis was utilised in the presence of JSRV, it could be hypothesized that this also may be the mechanism utilised by NPA to induce mitogenicity in very young lambs. The comparative data generated from the mannose inhibition study indicated that the immune status of JSRV-infected lambs, from the age of ten weeks, was more analogous to the immature immunological state identified in younger lambs, than would be ordinarily be anticipated for lambs of that age.

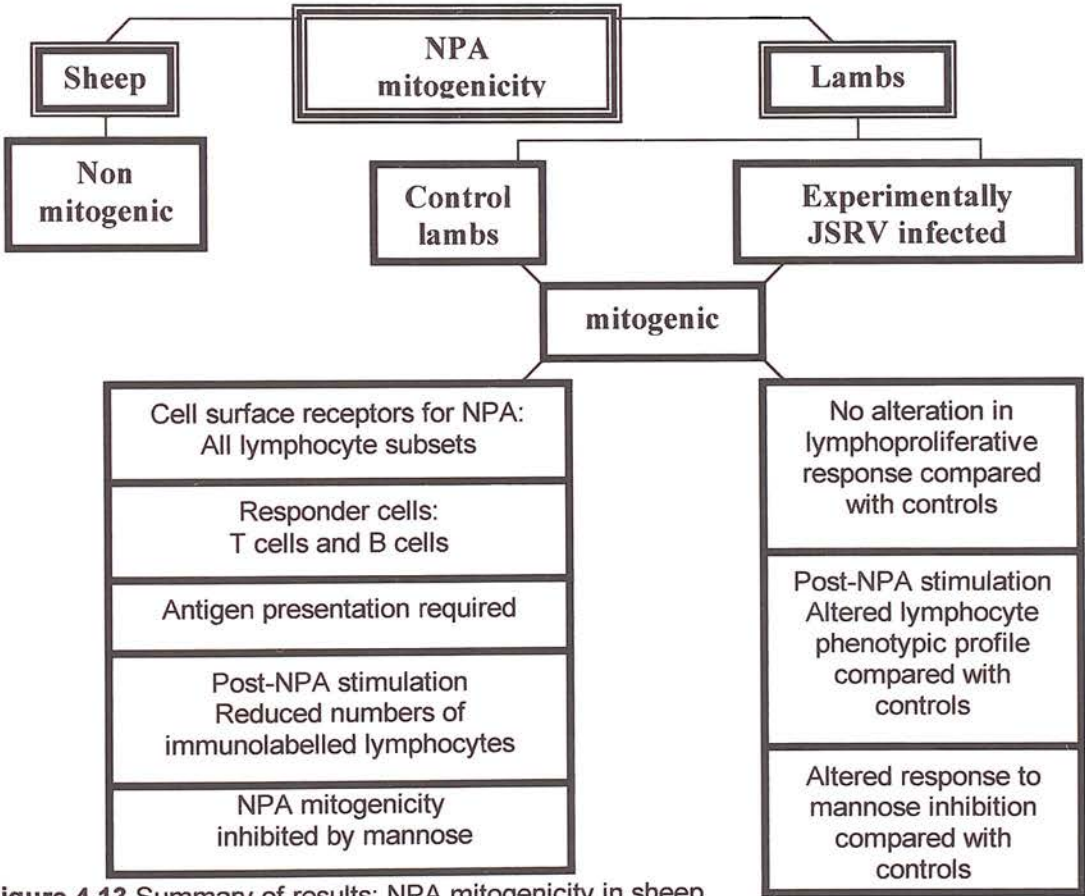


Figure 4.13 Summary of results: NPA mitogenicity in sheep

This study has revealed for the first time that the mitogenicity of the monocotyledonous lectin, NPA is age-related in humans (section 4.6), (Figure 4.12). In contrast to previous reports (Kilpatrick *et al* 1990, Fenton *et al* 1999) this study has shown that NPA is mitogenic for human lymphocytes. Clear evidence demonstrated that the lymphoproliferative response to NPA stimulation increased more than sevenfold for lymphocytes from umbilical cord blood, compared with the blood from adults (section 4.6.3). The lymphoproliferative responses to NPA in sheep reflected our observations in human lymphocytes (section 4.1.2). With no age-dependent response to PHA stimulation evident in either humans or sheep, these observations indicate that age-related divergence in the mononuclear cell population affects the mitogenicity of NPA.

Our findings relate only to *in vitro* studies and cannot predict *in vivo* responses to NPA. However, these results indicate that exposure of the human population to NPA could have physiological consequences. Research into the effects of the NPA-related *Galanthus nivalis* lectin (GNA) on human blood cells demonstrated the presence of many cell surface receptors (Fenton *et al* 1999). Consequently the lectin binding to the cell, if not culminating in lymphoproliferation may still have the potential to inhibit biological function. It also was proposed that glycosylation of white blood cells could result in increased synthesis of α 1-3-linked terminal mannosyl residues, the recognised binding site for both NPA and GNA. The PBMC examined were purified from frozen buffy coats (Fenton, personal communication) recovered from blood samples obtained exclusively from people over 60 years of age, thus the effects of NPA on the blood cells of younger human subjects remained un-addressed.

Following the discovery by Barker *et al* (1969) that poke weed mitogen (PWM) is mitogenic for human lymphocytes *in vitro* and *in vivo*, subsequent research has established that mitogenic plant lectins are able to withstand the degradative forces of the human and mouse digestive system, inducing mitogenic activity both within the gut and also systemically (Brady *et al* 1978, Kilpatrick *et al* 1985, Freed *et al* 1987). It is essential therefore that we understand the potential implications of introducing foreign lectins into human and animal food sources, and extend our knowledge of the plant lectin-mammalian cell relationship, not only for species specificity but also to include all age groups.

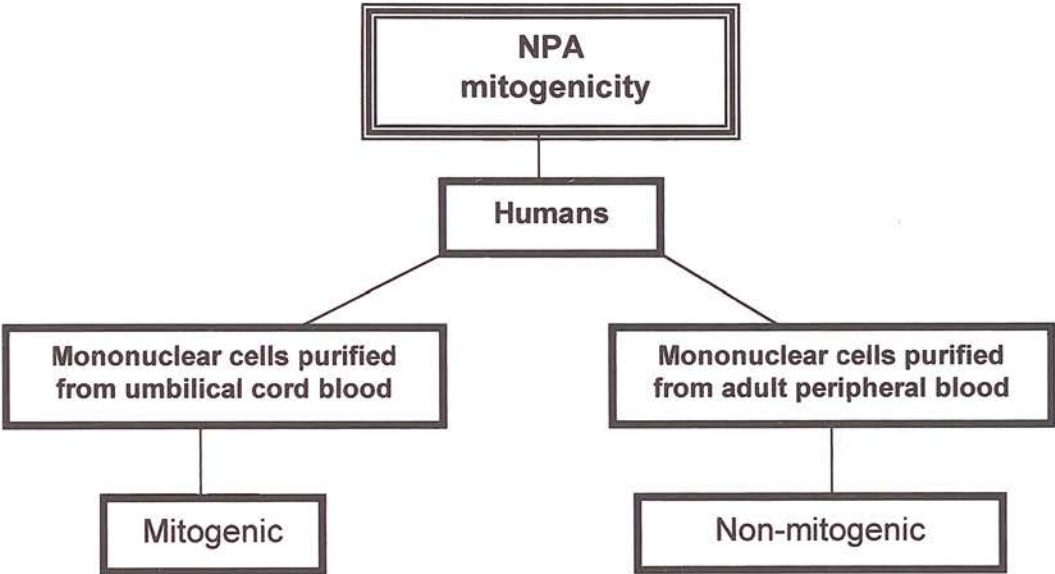


Figure 4.14 Summary of results: NPA mitogenicity in human subjects

Chapter 5

***In vitro* phenotyping of the OPA lung**

Introduction

Immunohistochemistry (IHC) is an *in situ* technique that allows characterization of different immune cells in histological sections, a distinction not possible with haematoxylin and eosin (H&E) staining. There are several immunohistochemical procedures and these have been extensively researched, adapted and employed for many purposes including diagnostics and *in situ* research into the pathogenesis of infections. Utilizing the specificity of monoclonal antibodies, the identification, distribution patterns and morphological changes in immune cells in infected tissues can be analysed and compared with tissues obtained from uninfected animals.

The main problem to be addressed in tissue fixation for subsequent immunolabelling is to establish a compromise between the retention of cellular morphology and tissue architecture, and the preservation of sensitive cell surface antigens. In this study a tissue fixation procedure was developed with the use of a fixative based on salts from a transition metal. Protocols were optimised for the preservation of sheep lung and lymph node, and for subsequent IHC of those tissues. Several fixation protocols were compared to test the quality of the post-fixed cellular morphology and the retention of sensitive antigens.

The conventional aldehyde-based fixatives (primarily formaldehyde, paraformaldehyde and their derivatives) undoubtedly retain the morphology of the fixed tissue with only minimal shrinkage. As a non-coagulating fixative, formaldehyde fixed tissues are not distorted by the generation of coagulum and the resultant production of tissue clumps. Formaldehyde, when dissolved in aqueous solution hydrates to form methylene glycol (Le Botlan *et al* 1983) so that this and

unhydrated formaldehyde rapidly penetrate through the tissue and form covalent bonds with highly reactive functional groups linking adjacent proteins, glycoproteins, polysaccharides or reactive sites such as primary amines (e.g. lysine) and thiols (e.g. cysteine). Once bound, these reactive molecules cross-link with the less reactive groups, such as amide or hydroxyl groups and cyclic aromatic rings, resulting in tissue fixation (Kunkel *et al* 1981). The methylene glycol/formaldehyde ratio in solution is maintained by dissociation of methylene glycol to formaldehyde resulting in a rapid penetration of the tissues paralleled by slow fixation. Therefore tissues fixed in formaldehyde do not become 'overfixed'. The major disadvantage of this fixation mechanism is directly related to the creation of the methyl bridges and cross-linking of the cells. This results, in many instances, in 'masking' the cell surface antigens, impeding subsequent immunolabelling. In order to reveal the antigens for IHC, the cross-links require to be broken and the cell surface epitopes revealed, without damage to the cells or to the tissues. The main strategies employed in antigen retrieval are protease digestion or heat activated degradation of the cross-linked proteins and/or molecules surrounding the antigenic site. Enzymatic digestion as a method of antigen retrieval was first reported in the 1960s and since then the use of proteolytic enzymes such as trypsin, pronase and pepsin has been employed with varying degrees of success (Gown *et al* 1993). The use of extreme temperatures to expose antigens has become widely used, principally by microwave (Shi *et al* 1991, Cattoretti *et al* 1993, Cattoretti and Suurmeijer 1995) or pressure cooker methods (Norton *et al* 1994). The different methods of antigen retrieval have been compared and evaluated (Cattoretti *et al* 1993, Miller *et al* 1995, Leoncini and Falini 1997, Pileri *et al* 1997) and many adaptations have been developed; temperature variation,

alterations in pH (Evers and Uylings 1994), comparison of buffers (Cattoretti and Suurmeijer 1995, Leong 1996), holding times (Evers and Uylings 1994), modification of the fixative, embedding procedures and tissue processing protocols (Beckstead 1985, Sato *et al* 1986). The fundamental problem in antigen retrieval is that no universal method exists. The variation in antigen sensitivity, monoclonal antibody efficacy and the ability of different tissues to withstand such severe treatment mean that no single method is applicable for all IHC.

As with the fixation method, the embedding procedure can also result in distortion and degradation of sensitive antigens. After the tissues have been fixed they are dehydrated and embedded in a matrix such as paraffin wax. This fortifies the tissue into a solid block, which allows thin sections to be cut and placed on glass slides for subsequent procedures. The use of plastic to embed tissues is employed, but paraffin wax is still the most universally used medium. The problem is exposure to high temperatures during the process. This has resulted in the development of paraffin waxes with melting temperatures as low as 50-60⁰C, which will not adversely affect heat sensitive antigens.

Cryopreservation is a technique also widely used for the preservation of routine tissue sections. Tissues collected at necropsy are encased in a cryomatrix, which protects the fresh tissue from the extreme temperature of snap freezing in liquid nitrogen and allows ease of handling. The tissues can be stored at -70⁰C until required. Relatively thin sections can then be cut on a cryostat. This method, in many instances, results in better preservation and reactivity of the cell surface antigens in comparison with aldehyde-fixed tissues. The frozen tissue blocks do not need to be embedded in paraffin wax, therefore any adverse effects resulting from this

procedure and the subsequent dewaxing process are eliminated. There is no cross-linking, therefore no antigen retrieval is necessary. Unfortunately this does not imply that freezing is the ideal method of tissue preservation. The major disadvantages of this procedure are that tissue may be adversely effected by freeze and thaw, with crystallisation within the cells disrupting the cellular morphology and the cell surface antigens. The actual procedure of cutting tissue sections with a cryostat is also extremely temperature sensitive and the temperature must be adjusted depending on the type of tissue to be cut. The optimum temperature must thereafter be maintained during the cutting process. Furthermore, cutting consistently uniform sections requires a great deal of skill. Some dense and compact tissues such as lymph nodes cut extremely well on a cryostat, whilst others such as lung are difficult to cut, with the tissue tearing as a result of the open network and large air spaces in the alveoli. Several sections are likely to be cut and discarded before a section of acceptable quality is produced.

To overcome some of the problems stated above, and establish an acceptable compromise between cellular morphology and antigen preservation, an alternative method of tissue fixation has been developed. In 1994, Beckstead reviewed the problems encountered by different tissue fixation methods and reported that the use of an aldehyde-free fixative based on salts of transition metals such as zinc resulted in excellent cellular morphology whilst preserving sensitive antigens. It was reported that the morphology of tissues preserved in zinc salts fixative (ZSF) was almost comparable with those fixed in aldehydes, while the survival of sensitive antigens was analogous to cryopreservation. Although the principles of this fixation method have been tested, the use of ZSF is not widespread and ZSF has not been tried as a

primary fixation method. It was hypothesised therefore that ZSF might provide the solution to the dilemma of morphology *versus* antigen preservation in the ovine species. In order to test this hypothesis, an investigation into the properties of ZSF as a fixation agent for sheep tissue was undertaken. Adaptations of the original ZSF method, particularly temperature ranges and fixation times were optimised. The subsequent immunolabelling of tissues, which had been aldehyde fixed, cryopreserved and fixed in ZSF, was compared.

It was proposed that, once established, the optimal fixation method could be used to preserve OPA tumour and non-neoplastic lung tissues collected at necropsy from confirmed OPA cases and uninfected control lambs for subsequent IHC to determine the identity of tumour infiltrating cells and examine the distribution and arrangement of immune cells.

5.1 Comparison of tissues fixed in zinc salts fixative (ZSF), aldehydes and cryopreserved sections.

5.3.1 Methodology

To compare the quality of cellular morphology and immunolabelling in ZSF-fixed tissues with tissues subjected to other fixatives, samples (5-6mm) of lung, bronchial, mediastinal and popliteal lymph node were collected at necropsy from experimentally JSRV-infected lambs and adult OPA cases (Chapter 2, sections 2.1.5 and 2.1.1, respectively). The tissues were fixed in ZFS (Chapter 2, section 2.10.1), 4%, 2% and 1% formaldehyde and 4% paraformaldehyde (Chapter 2, section 2.10.2) and subsequently paraffin wax embedded as detailed in Chapter 2, section 2.11.1. Sections were cut from the preserved tissue blocks and antigen retrieval was attempted with each of the aldehyde-fixed sections (Chapter 2, section 2.11.3).

Tissue samples were also cryopreserved (Chapter 2, section 2.10.4) and sections cut and prepared for IHC (Chapter 2, section 2.12). Immunolabelling was preformed (Chapter 2, section 2.12) using the panel of monoclonal antibodies detailed in Table 5.1, at the dilutions shown. The EnVision kit (DAKO, Ely, Cambridge, UK) described in Chapter 2, section 2.13.1 was the detection system used.

| Mab | Antigen | Reactivity | Dilutions tested |
|--------|--------------------|------------------------------|-----------------------------|
| VPM5 | CD1 | Dendritic cells | 1:50, 1:100, 1:500 |
| 135/A | CD2 | T cells [$\alpha\beta$] | 1:50, 1:100, 1:500, 1:800 |
| 17D | CD4 | T _{HELPER} cells | 1:50, 1:100, 1:500 |
| SBU-T8 | CD8 | T _{CYTOTOXIC} cells | 1:50, 1:100, 1:500 |
| 86D | $\gamma\delta$ TCR | $\gamma\delta$ T cells | 1:50, 1:100, 1:500 |
| VPM8 | Anti-light chain | B cells | 1:100, 1:500, 1:800, 1:1000 |
| VPM65 | CD14 | Monocytes/macrophages | 1:50, 1:100, 1:500 |

Table 5. 1 Panel of monoclonal antibodies for immunohistochemical comparison of tissues preserved in 4%, 2% and 1% formaldehyde, 4% paraformaldehyde, cryopreserved or fixed in ZSF. All monoclonal antibodies were from cell culture supernatants.

5.1.2 ZSF is the preferred fixation method for immunohistochemistry of ovine lung and lymph nodes

Formaldehyde fixation: In tissues fixed in formaldehydes, no positive cells were detected in sections labelled with any of the dilutions of monoclonal antibodies specific for CD1, CD2, CD4 and CD8 T cells. Immunolabelling to detect $\gamma\delta$ T cells, B cells and macrophages did result in some non-specific labelling, with a reticular staining pattern observed throughout the tissue. Antigen retrieval by pressure cooker

or microwave oven methods (Chapter 2.11.3) did not improve the quality of the immunolabelling. Several attempts at antigen retrieval, altering the conditions by increasing the incubation times and trying different buffers as detailed in Chapter 2, section 2.11.3 did not enhance the results.

Cryopreservation: The quality of the tissue morphology was not preserved to an acceptable standard in the cryopreserved lung sections. No positive immunolabelling was detected with any of the monoclonal antibodies tested at 1:50 dilution. Monoclonal antibodies recognising CD1, CD4 and CD8 T cells resulted in positive labelling at 1:100 dilution but a large amount of non-specific background labelling was also evident, and at higher dilutions no positive immunolabelling was detected. In sections labelled for the detection of $\gamma\delta$ T cells, macrophages and in particular B cells, the level of background staining was very high at 1:100 dilution, and no positive labelling was detected at 1:500 dilution. Attempts to decrease the background level by increasing the incubation time of the peroxidase block and removal of non-specific antigens by increasing the incubation time of the goat serum did not enhance the quality of the labelling. No positively immunolabelled CD2 T cells were detected with any dilution of the monoclonal antibody tested.

Zinc Salts Fixative: In contrast to the other methods tested, immunolabelling of ZSF tissues resulted in improved detection of discrete positively labelled cells whilst retaining cellular morphology. Immunolabelling ZSF tissues with the monoclonal antibodies recognising CD1, CD4, CD8, $\gamma\delta$ T cells and macrophages resulted in the detection of positively labelled discrete cells, at 1:100 dilution. However, immunolabelling for the detection of CD2 T cells did not result in positive labelling, at any dilution of the monoclonal antibody tested. In the detection of B

cells, high levels of non-specific background labelling was evident up to 1:100 dilution of the monoclonal antibody VPM8 and no positive labelling was detected at higher dilutions (results not shown).

5.2 Optimisation of ZSF for the preservation of ovine tissues.

5.2.1 Methodology

To determine the optimum conditions for the preservation of ovine tissue in ZSF, samples (5-6mm) of bronchial, mediastinal, popliteal and prescapular lymph node, lung tumour and non-neoplastic lung were collected at necropsy from experimentally JSRV-infected lambs and adult OPA sheep (Chapter 2, sections 2.1.5 and 2.1.1, respectively). The tissues were fixed in ZSF (Chapter 2, section 2.10.1) at room temperature and at 37⁰C, and in alcohol/zinc acetate (AZA) (Chapter 2, section 2.10.2) at room temperature. After primary fixation of 4 – 6 hours, the tissues were trimmed to 2-3mm thick sections and post-fixed (in cassettes) in a fresh volume of the same fixative and temperature as detailed above, for 24 hours. The tissues were then dehydrated and paraffin wax embedded as detailed in Chapter 2, section 2.11.1.

5.2.2 Cellular morphology and immunolabelling are optimal in ZSF, at room temperature and 37⁰C

The quality of the cellular morphology and subsequent immunolabelling (detailed in Chapter 2, section 2.12) for each fixation protocol was compared. Tissues fixed in AZA resulted in a poor standard of cell morphology. Tissues preserved in ZSF at room temperature and 37⁰C were found to be comparable, resulting in acceptable cellular morphology and clear immunolabelling. This preliminary work was performed by Karen Neligan (MSc Project Thesis, Napier University, Edinburgh) under the supervision of Dr. Lorenzo Gonzalez (Veterinary

Laboratory Agencies, Penicuik, Midlothian. Scotland). The investigation was extended to adapt and further develop the fixation process with the results detailed in the publication at the end.

5.3 Optimisation of monoclonal antibodies for immunolabelling ovine tissue

5.3.1 Methodology

The availability of different mouse anti-sheep monoclonal antibody clones was investigated for the compilation of a panel of monoclonal antibodies, which would result in the best quality immunolabelling of ZSF tissues. To determine the optimum conditions for immunohistochemistry with ZSF tissues, it was necessary to optimise the concentration of each monoclonal antibody selected.

Samples (5-6mm) of caudal, mediastinal, tracheobronchial and popliteal lymph nodes were collected at necropsy and fixed in ZSF (Chapter 2, section 2.10.1). The tissue sections were dewaxed, rehydrated and immunolabelled as detailed in Chapter 2, section 2.13, using the panel of monoclonal antibodies outlined in Table 5.2. In addition, the monoclonal antibodies SBU-TcII specific for MHC class II, VPM30 specific for B cells and activated T cells, and VPM8 specific for B cells were tested also. Various dilutions, ranging from 1:25 to 1:800 for monoclonal antibodies from cell culture supernatants and 1:1000 to 1:256 000 for ascites fluid monoclonal antibodies, were tested. Incubation at room temperature for 30 minutes and overnight at 4°C was compared. The EnVision detection system (secondary antibody, peroxidase-labelled polymer conjugated to goat anti-mouse immunoglobulin) was applied as detailed in Chapter 2, section 2.12. The immunolabelled sections were microscopically examined, and the optimum dilution

was determined based on the quality of discrete immunolabelled cells and low levels of background staining. These dilutions are shown in Table 5.2.

5.3.2 At optimum monoclonal antibody concentrations, a high standard of immunolabelling and cellular morphology was demonstrated in ZSF lymph nodes

The quality of cellular morphology was retained with all the sections examined at the optimum monoclonal antibody concentrations (Table 5.2). Precise definition of discrete immunolabelled cells was demonstrated within the lymph nodes, in accordance with the reported reactivity and cell-specificity previously demonstrated by the authors referenced in Table 5.2. CD1, CD4 and CD8 epitopes, regarded as highly sensitive to aldehyde-fixation and cryopreservation (Beckstead 1994, and section 5.3.2) were demonstrated in ZSF tissues. In addition, immunolabelling of more resistant epitopes such as WC-1, MHC class II, CD14 and CD21 was enhanced in ZSF tissues. The monoclonal antibodies VPM8 and VPM30 did not detect B cells in the germinal centres of the lymphoid follicle but did strongly label cells in the medullary cords that resembled plasmacytes (Gonzalez *et al* 2001, see publication at end of thesis). The optimisation of the monoclonal antibodies was performed, after discussion, by Ian Anderson (Moredun Research Institute), with the results analysed by Dr. Lorenzo Gonzalez (Veterinary Laboratory Agencies, Penicuik, Midlothian. Scotland).

5.4 Immunohistochemistry of ZSF sheep lymph nodes, and lung tissues from lambs with experimentally induced OPA and uninfected controls.

To determine any alteration in the distribution pattern of immune cells in the OPA lung, compared with uninfected lung and to characterise the subtypes and

| Mab | Antigen | Reactivity | Optimum dilution | Source | Reference |
|---------|---------|-----------------------------------|------------------|-----------|----------------------------|
| 17D | CD4 | T _{HELPER} cells | 3 000 AF | Basel | Maddox <i>et al</i> (1985) |
| SBU-T8* | CD8 | T _{CYTOTOXIC} cells | 100 SN | Melbourne | Maddox <i>et al</i> (1985) |
| CC15 | WC-1 | $\gamma\delta$ T cells | 100 000 AF | Compton | Howard <i>et al</i> (1989) |
| CC21 | CD21 | FDCs | 30 000 AF | Compton | Nacssens & Howard (1991) |
| CC20 | CD1b | Mature B cells Dendritic cells | 10 000 AF | Compton | Howard <i>et al</i> (1993) |
| VPM65 | CD14 | Monocytes macrophages | 5 000 AF | Edinburgh | Gupta <i>et al</i> (1996) |
| VPM 20 | IgG1 | Non-specific control | 1:1000 AF | Edinburgh | |
| VPM 22 | IGG2 | Non-specific control | 1:1000 AF | Edinburgh | |

Table 5.2 Monoclonal antibodies tested for immunolabelling sheep lymph nodes and lungs fixed in ZSF and paraffin wax-embedded. Basel: Basel Institute for Immunology, Basel Switzerland. Compton: BBSRC Institute for Animal Health, Compton Laboratory, Newbury, Berks, UK. Edinburgh: Veterinary pathology, University of Edinburgh, UK. Melbourne: University of Melbourne, School of Veterinary Science, Parkville, Victoria, Australia. CD, cluster designation; WC-1, workshop cluster 1; FDCs, follicular dendritic cells; AF, ascites fluid; SN, monoclonal antibodies from culture supernatant. * Clone 38-65.

location of tumour infiltrating leukocytes, samples of OPA tumour and non-neoplastic lung were collected at necropsy from three specific pathogen free (SPF) lambs with experimentally induced OPA and three SPF uninfected control lambs. OPA lesions were macroscopically detected in the lungs of two lambs and microscopic lesions were identified in the third lamb. Histopathological analysis of lung tissue from all three lambs confirmed that the lesions were OPA.

5.4.1 Methodology

Sections 5-6mm thick of macroscopically visible areas of tumour and sections of lung from the lamb with microscopic nodules, together with lung tissue from the control lambs were collected at necropsy. The tissues were fixed in ZSF and paraffin wax embedded for subsequent immunolabelling. The tissue sections were

cut and stored at 4⁰C for a maximum of seven days prior to immunolabelling. IHC was performed as detailed in Chapter 2, section 2.13 using the panel of monoclonal antibodies and controls outlined in Table 5.2, at the previously optimised dilutions shown. In addition to the lung sections, immunohistochemistry was performed on sections of sheep lymph node to confirm that the location of the immunolabelled cells within the lymph node was in accordance with the previous report (Gonzalez *et al* 2001).

5.4.2 Immunohistochemistry to detect cell populations in sheep lymph nodes

CD4 T cells: The monoclonal antibody 17D was used to detect CD4 T cells within the sheep lymph node. It was revealed that cells expressing this epitope were morphologically small and spherical, distributed as small clusters or as individual cells. The positively immunolabelled population was predominantly located in the paracortex of the lymph node, contiguous with the periphery of the follicles. In addition some individual immunolabelled cells were observed within the follicles (Figure 5.1).

CD8 T cells: The monoclonal antibody SBU-T8, used to detect CD8 T cells revealed that cells expressing this epitope were small, spherical and located in clusters or as individual cells. They were primarily distributed in the paracortical region of the lymph node adjacent to the follicles, in contrast to CD4 T cells there was very little evidence of positively immunolabelled CD8 T cells within the follicles (Figure 5.2).

$\gamma\delta$ T cells: The WC-1 epitope was detected by immunolabelling with the monoclonal antibody CC15 identifying $\gamma\delta$ T cells within the sheep lymph node. Small, spherical cells were detected throughout the paracortex, distributed as

scattered individual cells. Some cells were identified lining the subcapsular and trabecular sinuses, and the population of positively immunolabelled cells appeared to intensify at the capsule/cortical junction (Figure 5.3).

Dendritic cells: The epitope CD1b was detected by immunolabelling with the monoclonal antibody CC20, identifying dendritic cells within the lymph node. The immunolabelling was very faint and the morphology of the individual cells was difficult to distinguish. All the positively immunolabelled cells detected were found in the paracortex, around the follicles (Figure 5.4).

Follicular dendritic cells and mature B cells: The monoclonal antibody CC21 recognises the epitope CD21, found on follicular dendritic cells and mature B cells. The positively immunolabelled cells within the sheep lymph node were detected almost exclusively within the light zone of the germinal centres within the follicles (Figure 5.5). The location and morphology of these cells, when seen at a higher magnification, were large with irregular shape and evidence of reticulated processes suggest that they are follicular dendritic cells.

Macrophages: The monoclonal antibody VPM65 is specific for the CD14 molecules found on monocytes and macrophages. Within the sheep lymph node the positively immunolabelled cells were identified, mainly as dispersed individual cells, in the medullary cords: No positively labelled cells were identified in the follicles and only very few cells were observed in the paracortex. Morphologically the cells were larger than lymphocytes and contained an extensive cytoplasm (Figure 5.6).

Summary of results: Immunolabelling was consistent with the findings previously reported by Gonzalez *et al* (2001), although the labelling of dendritic cells with the monoclonal antibody CC20 was not particularly clear with very few positive

cells detected. The distribution of the lymphocyte subsets within the lymph nodes corresponded with the previous report, including the location of $\gamma\delta$ T cells, indicating their transient passage through the lymph node (Hein and Mackay 1991). The cells detected by the monoclonal antibody CC21 morphologically appeared to be follicular dendritic cells, in accordance with the previous study. The location and distribution of macrophages in the lymph node also corresponded to the previous finding.

Figure 5.1. CD4⁺ T cells immunolabelled with MAb 17D. Positively immunolabelled cells were detected in the paracortex of the lymph node, with some individual, scattered cells located in the follicle. Immunohistochemistry (IHC) and haematoxylin counterstain. X 100.

Figure 5.2. CD8⁺ T cells immunolabelled with MAb SBU-T8. Positively immunolabelled cells were detected in the paracortex of the lymph node. IHC and haematoxylin counterstain. X 100.

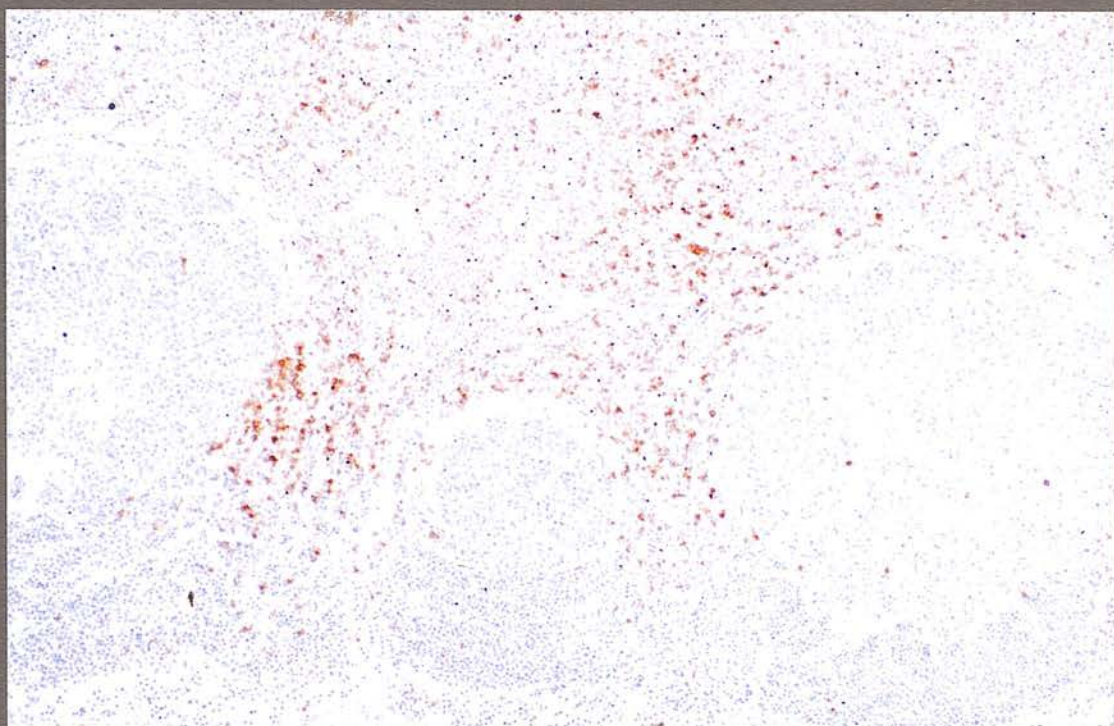
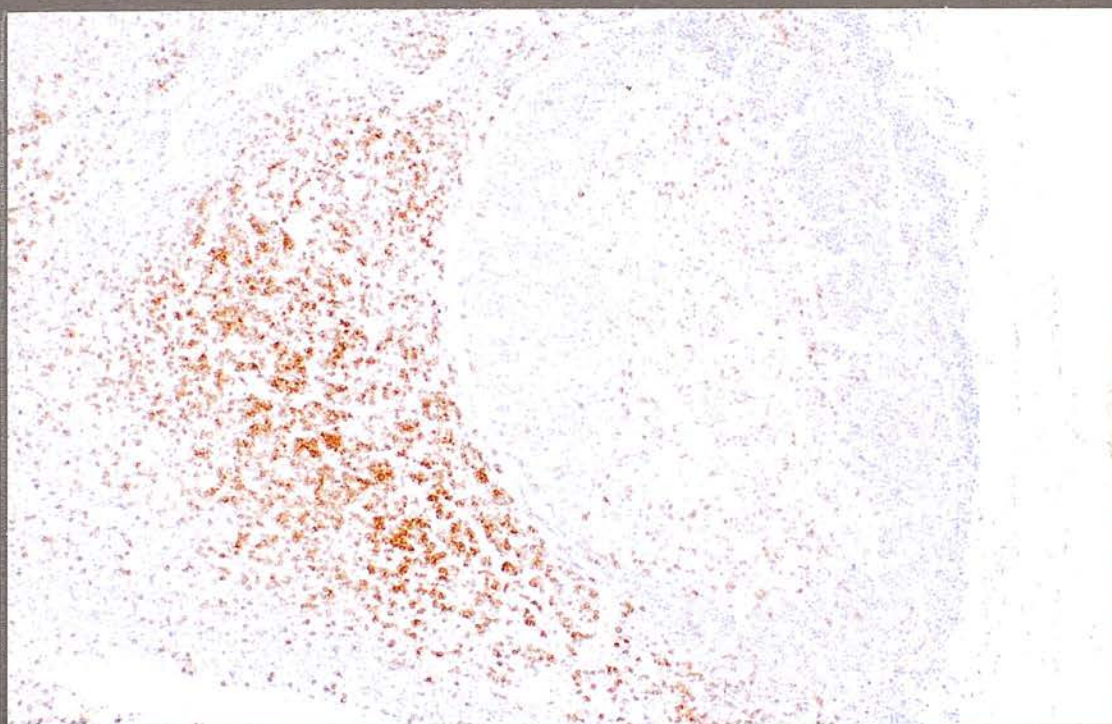


Figure 5.3. $\gamma\delta$ T cells immunolabelled with MAb CC15. Positively immunolabelled cells were detected in the paracortex of the lymph node, mainly at the capsule/cortical junction. IHC and haematoxylin counterstain. X 100.

Figure 5.4. Dendritic cells immunolabelled with MAb CC20. In the paracortex of the lymph node only a very few positively labelled cells were detected. IHC and haematoxylin counterstain. X 100.

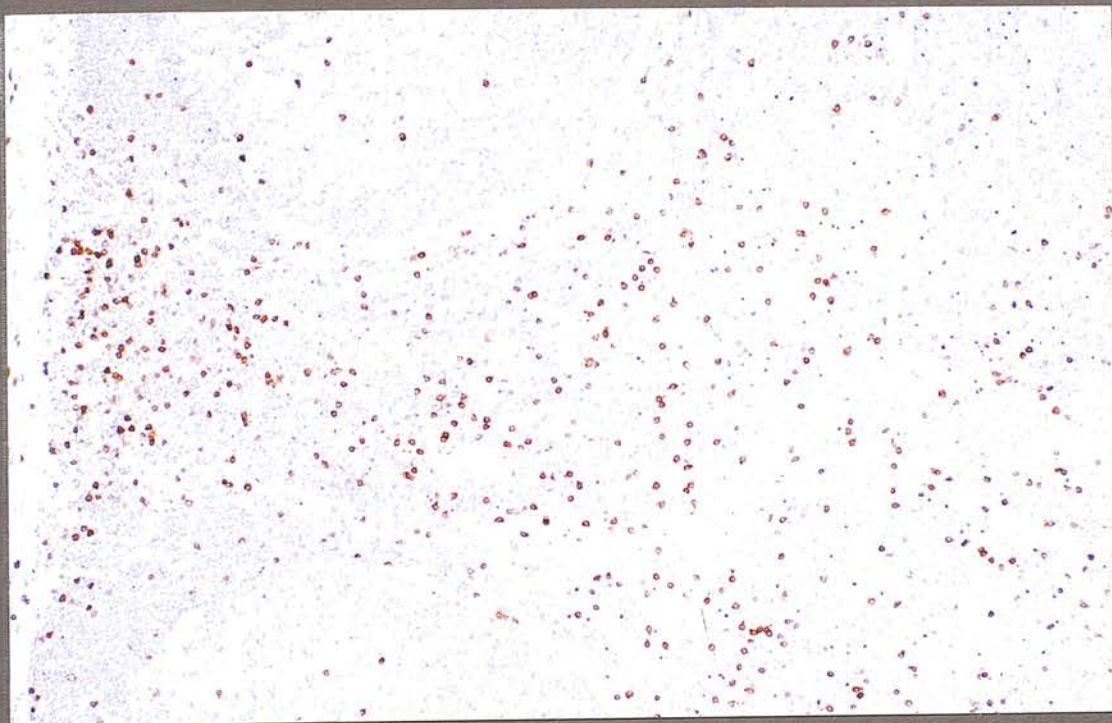
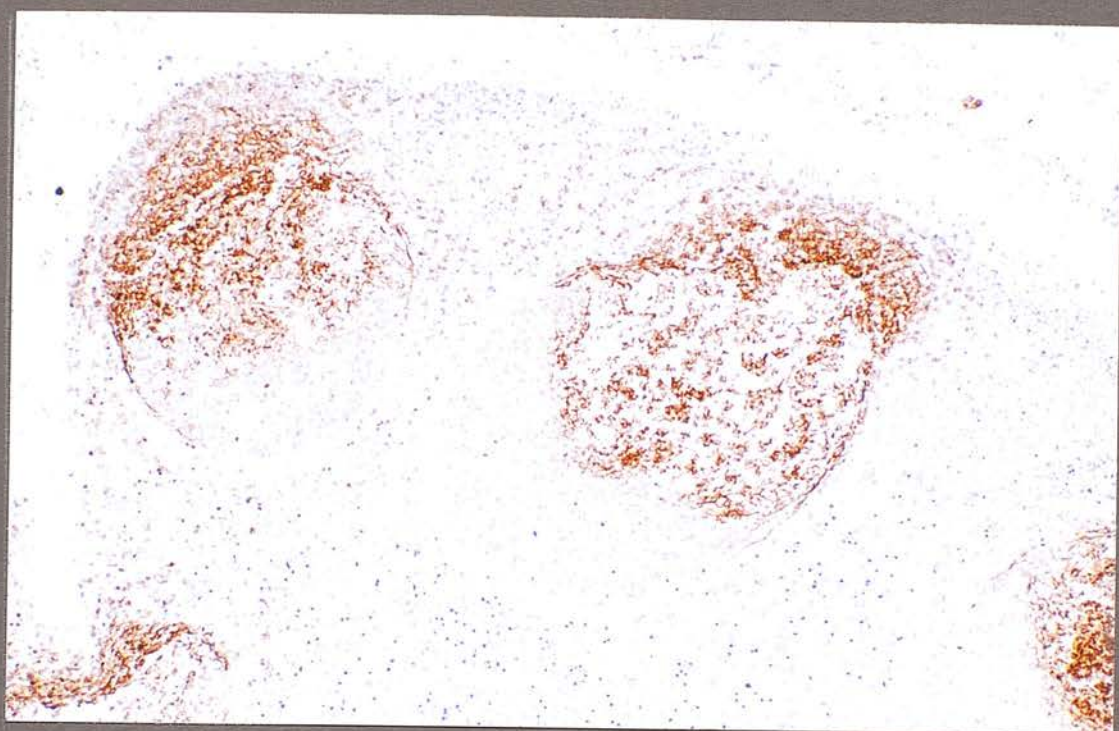


Figure 5.5. Mature B cells and follicular dendritic cells immunolabelled with MAb CC21, recognising the CD21 epitope. In the lymph node clusters of positively immunolabelled cells were detected in the light zone of the follicles. IHC and haematoxylin counterstain. X 100.

Figure 5.6 Macrophages immunolabelled with MAb VPM65. Positively immunolabelled cells with large pale nuclei and extensive cytoplasm were detected in the medullary sinuses of the lymph node. IHC and haematoxylin counterstain. X 400.



5.4.4 Immunohistochemistry to detect alterations in cell types and distribution patterns in OPA tumour, non-neoplastic lung and uninfected lung of SPF lambs

In situ immunolabelling was performed to compare the location and distribution of leukocytes in lung tissue from SPF uninfected lambs, the non-neoplastic area of lung and tumour from SPF lambs with confirmed OPA.

CD4 T cells: In both the uninfected lung and non-neoplastic region of the OPA lung, CD4 T cells detected by the monoclonal antibody 17D were located mainly in the bronchial associated lymphoid tissue (BALT). The immunolabelled cells were small, spherical and distributed individually or as small clusters as shown in Figure 5.7. Some positively immunolabelled cells were detected in the alveolar walls, where they were distributed as individual cells (Figure 5.8).

In the OPA tumour sections, immunolabelled CD4 T cells were detected infiltrating the tumour but this was not consistent for all tumour nodules. Positively labelled cells were found in larger nodules, which were observed macroscopically as shown in Figure 5.9, but no CD4 T cells were detected in microscopic nodules. The cells were small and spherical and no particular distribution pattern was evident. There was no evidence of cell migration to the tumour site (Figure 5.9).

CD8 T cells: A considerable population of CD8 T cells, identified by the monoclonal antibody SBU-T8, was detected in the peripheral region of the BALT from the uninfected lung and in the non-neoplastic region of the OPA lung. Morphologically the CD8 T cells were small and spherical, and were distributed as small clusters or individual cells (Figure 5.10). Positively immunolabelled cells were also detected in the walls and lumen of the alveoli, where they were distributed

mainly as individual cells. This distribution pattern was found in the uninfected lung, and in the non-neoplastic regions of the OPA lung (Figure 5.11).

In large tumour nodules very few immunolabelled CD8 T cells were detected, and these cells were observed only around the periphery of the tumour with practically no evidence of infiltration. In microscopic OPA tumours, positively immunolabelled cells were detected in the stroma and localized to the periphery of the tumour, with only very few positively labelled cells detected infiltrating the centre. The small, spherical cells were distributed as scattered individuals rather than as groups. A microscopic tumour is shown in Figure 5.12.

$\gamma\delta$ T cells: A large population of $\gamma\delta$ T cells, identified by the monoclonal antibody CC15, was detected in the sections from the uninfected lung and the non-neoplastic region of the OPA lung. Large numbers of positively immunolabelled $\gamma\delta$ T cells were identified in the BALT and in the lamina propria of the bronchioles. Morphologically the $\gamma\delta$ T cells were small and spherical, and were distributed as small clusters or individual cells (Figure 5.13). Positively immunolabelled cells were also observed in the walls and particularly the lumen of the alveoli. In addition $\gamma\delta$ T cells were detected around the blood vessels. (Figure 5.14).

$\gamma\delta$ T cells were detected in the periphery of OPA tumour nodules, closely associated to the epithelium. There was no evidence of positively immunolabelled cells infiltrating the centre of the tumour. $\gamma\delta$ T cells were detected around small and large tumour nodules, distributed mainly as individual cells (Figure 5.15).

Dendritic cells: The monoclonal antibody CC20, specific for the CD1 epitope detected only few dendritic cells in the uninfected and OPA lung. Positively immunolabelled dendritic cells were detected in the airways and associated with the

BALT. Some positively labelled cells were identified around the walls of the blood vessels and bronchioles. Morphologically the cells were stellate or irregular in shape and were mainly distributed as individual cells (Figure 5.16). Positively immunolabelled cells were also detected in the alveolar lumen, and to a lesser extent, in the alveolar walls from both the uninfected lung and non-neoplastic region of the OPA lung (Figure 5.17).

Very few positively immunolabelled dendritic cells were detected in tumour nodules. Single cells were identified in the periphery of several tumour nodules. The detection of positively labelled cells was not dependent on the size of the tumour and no distribution pattern was evident (Figure 5.18).

B cells: The monoclonal antibody CC21, specific for the CD21 epitope recognised mature B cells and follicular dendritic cells in the uninfected and OPA lung. Large populations of small, spherical, positively immunolabelled cells were detected in the BALT (Figure 5.19). The labelled cells were localized in the centre of the BALT aggregate, inverse to where immunolabelled CD8 T cells were detected, as previously shown in Figure 5.12. Positively immunolabelled cells were also detected in the alveolar walls of both the uninfected lung and non-neoplastic region of the OPA lung (Figure 5.20).

There was no evidence of positively immunolabelled cells at the site of any tumour nodules examined. No positive cells were detected surrounding the periphery or infiltrating the tumour (Figure 5.21).

Macrophages: The monoclonal antibody VPM65 detected macrophages in the uninfected and OPA lung. In the uninfected lung, no positively immunolabelled cells were detected in the BALT and only a few positive cells were identified in the

alveolar lumen (Figure 5.22). In the non-neoplastic region of the OPA lung, many immunolabelled cells were identified in the BALT, in the connective tissue around major bronchii and in the alveolar lumen. The positively labelled cells appeared to be larger than those detected in the uninfected lung (Figure 5.23).

Positively immunolabelled cells were detected in the areas where tumour nodules were present. Macrophages were observed in the stroma around the tumours and in the alveolar lumen. Morphologically the cells were large and irregular in shape (Figure 5.24).

Summary of results: No differences in the lymphoid cell subsets, their localization and distribution patterns were found between the uninfected lung and non-neoplastic region of the OPA lung from SPF lambs, with the exception of macrophages. The very few macrophages detected in the uninfected lung were mainly confined to the BALT, whereas in the non-neoplastic region of OPA lungs macrophages were detected not only in the BALT but also in the parenchyma of the lung. In the OPA tumour there was very little evidence of tumour infiltration, with the majority of lymphoid subsets found around the periphery of the nodules and the surrounding stroma.

Figure 5.7. CD4⁺ T cells immunolabelled with MAb 17D in the uninfected lung from a control lamb. Positively immunolabelled cells were detected mainly in the bronchial associated lymphoid tissue (BALT). IHC and haematoxylin counterstain. X 250.

Figure 5.8. CD4⁺ T cells immunolabelled with MAb 17D in the non-neoplastic region of the OPA lung. Individual positively immunolabelled cells were detected in the alveolar walls. IHC and haematoxylin counterstain. X 200.

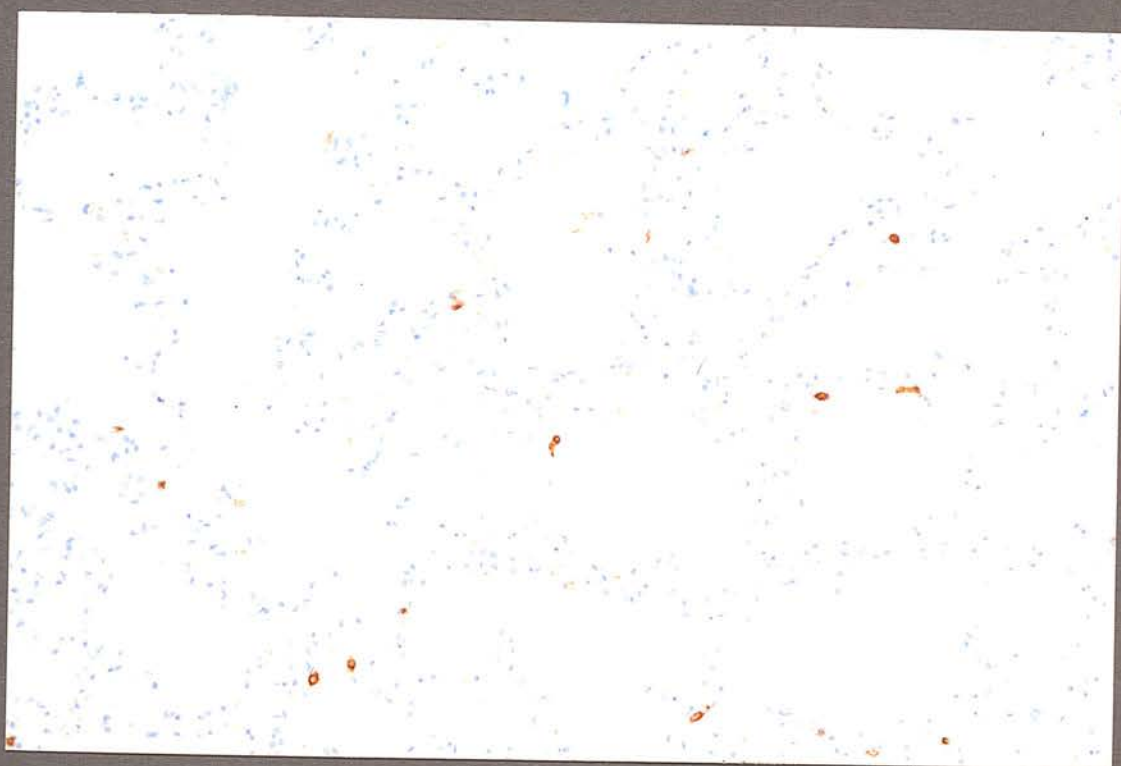
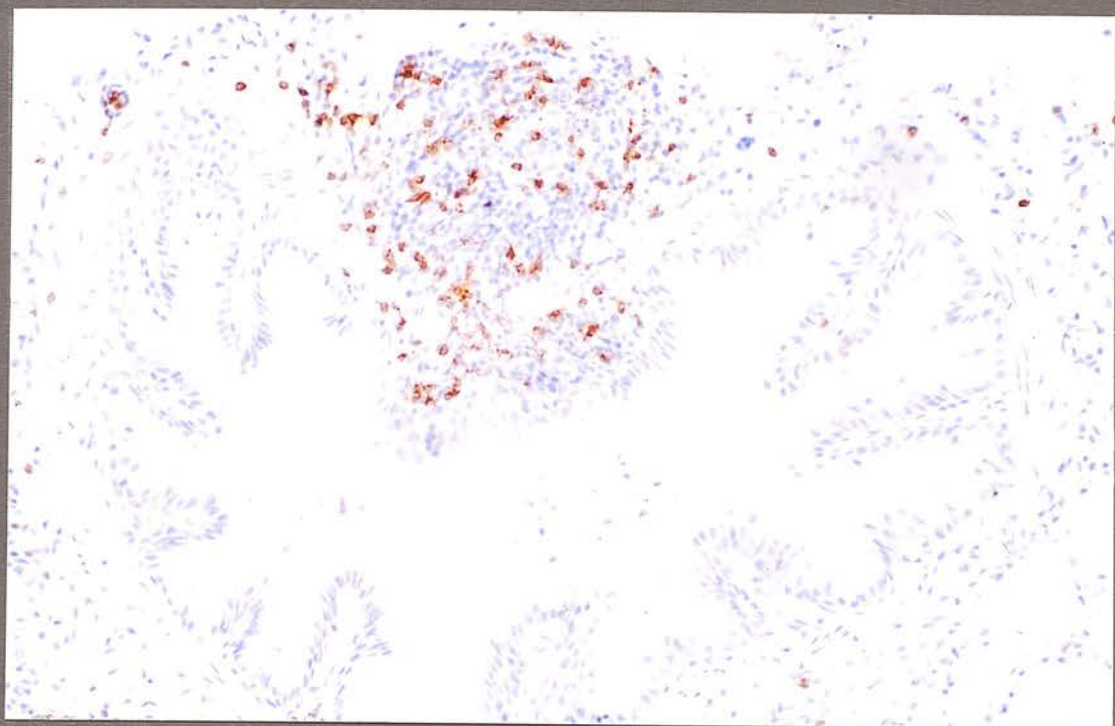


Figure 5.9. CD4⁺ T cells immunolabelled with MAb 17D in OPA tumour.
Positively immunolabelled cells were detected infiltrating the tumour nodule.
IHC and haematoxylin counterstain. X 400.

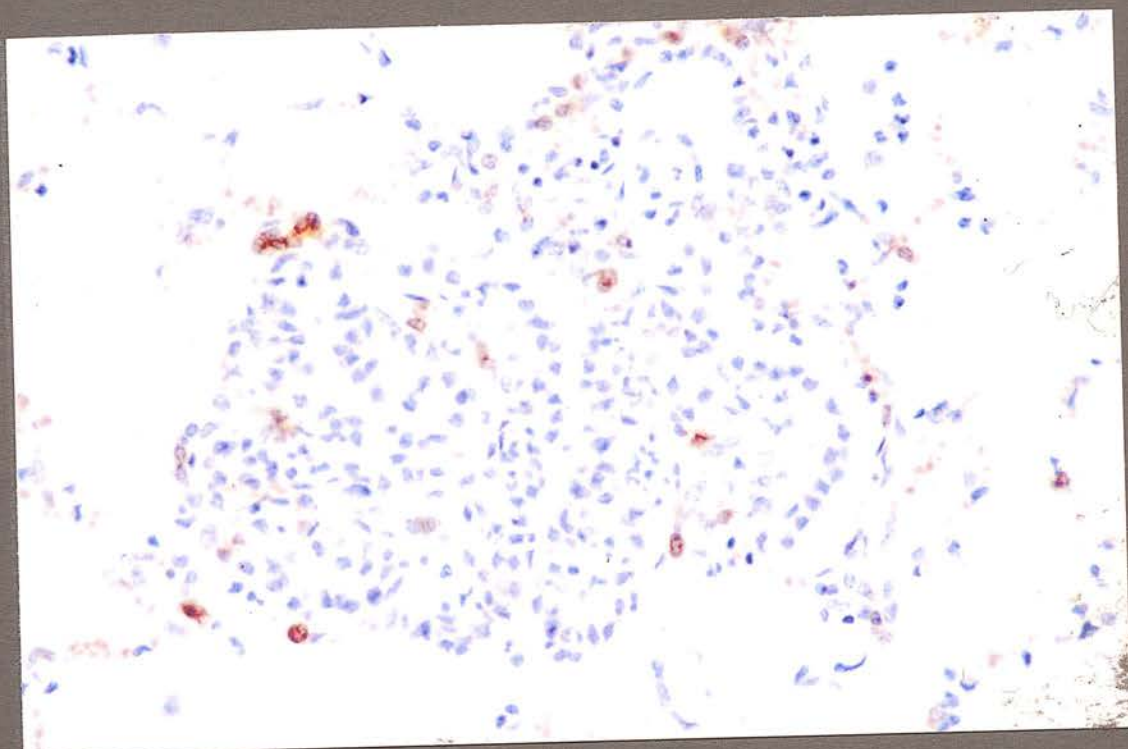


Figure 5.10. CD8⁺ T cells immunolabelled with MAb SBU-T8 in the uninfected lung from a control lamb. Positively immunolabelled cells were detected in the BALT. IHC and haematoxylin counterstain. X 100.

Figure 5.11. CD8⁺ T cells immunolabelled with MAb SBU-T8 in the non-neoplastic region of the OPA lung. Positively immunolabelled cells were detected in the alveolar walls and alveolar lumen. IHC and haematoxylin counterstain. X 400.

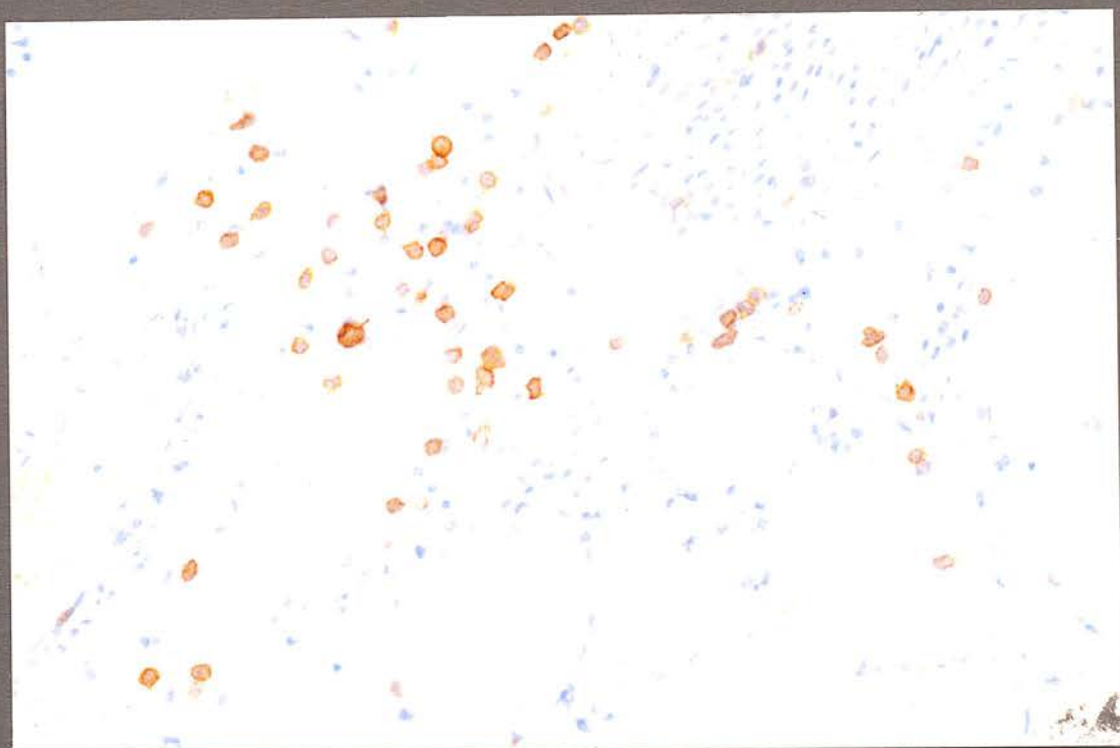
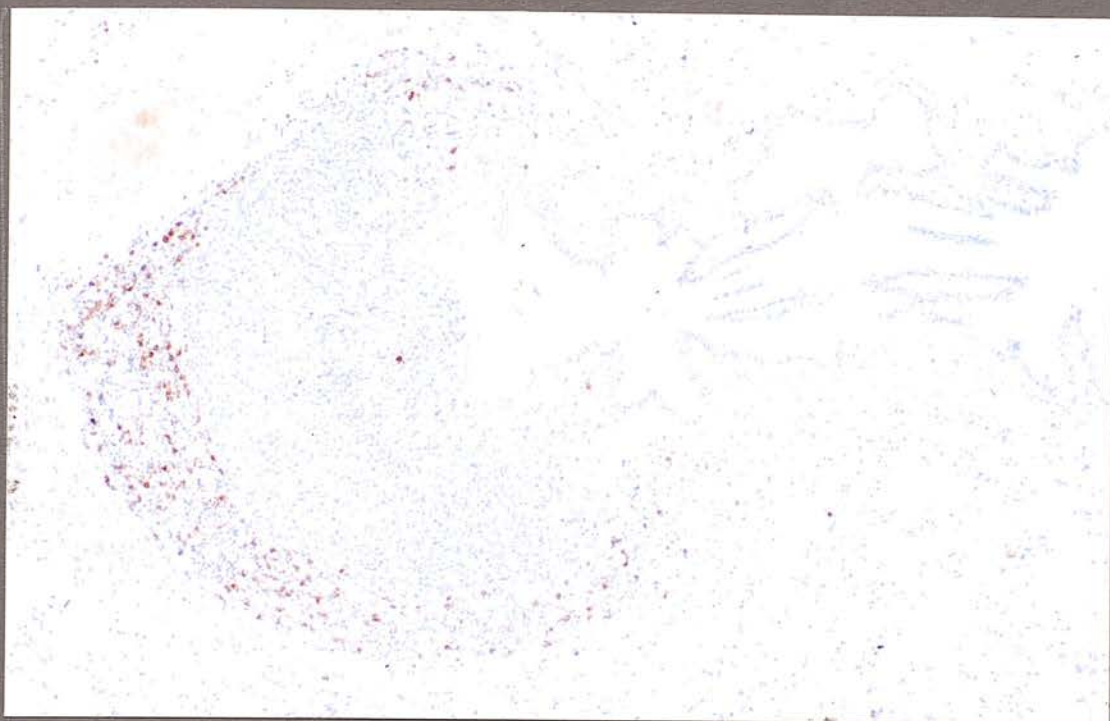


Figure 5.12. CD8⁺ T cells immunolabelled with MAb SBU-T8 in a microscopic OPA tumour. Positively immunolabelled cells were detected in the stroma and around the periphery of the tumour. IHC and haematoxylin counterstain. X 250.

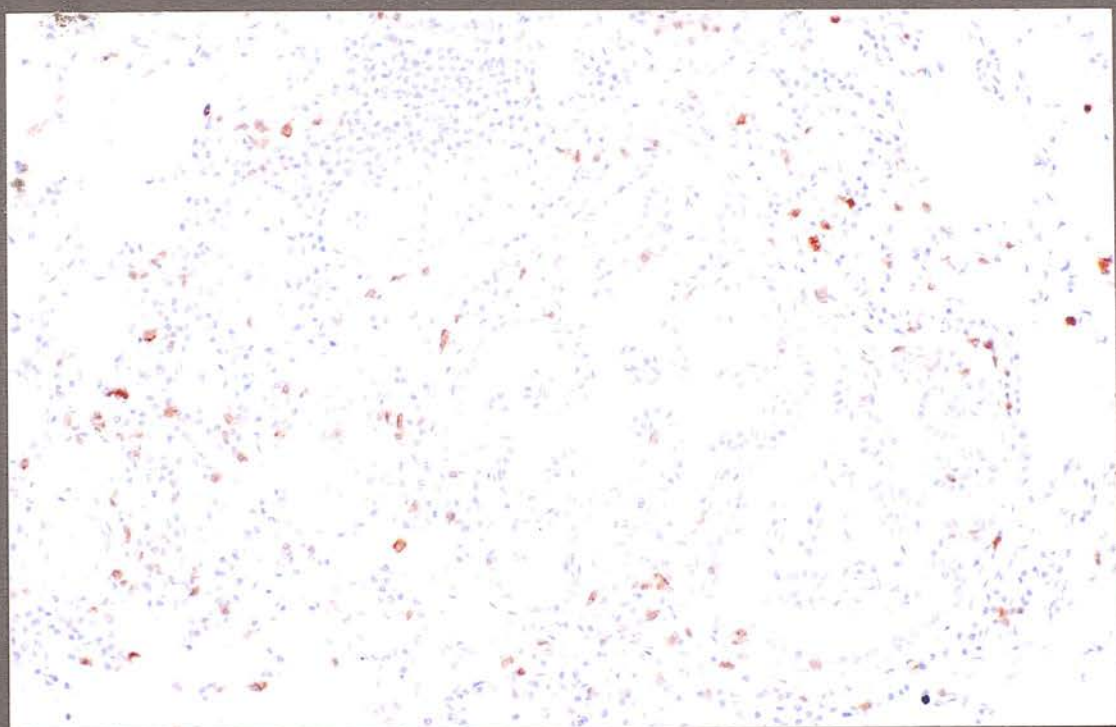


Figure 5.13. $\gamma\delta$ T cells immunolabelled with MAb CC15 in the uninfected lung from a control lamb. Positively immunolabelled cells were detected in the BALT and alveolar walls. IHC and haematoxylin counterstain. X 250.

Figure 5.14. $\gamma\delta$ T cells immunolabelled with MAb CC15 in the non-neoplastic region of the OPA lung. Positively immunolabelled cells were detected in the alveolar walls, lumen and lining the blood vessels. IHC and haematoxylin counterstain. X 200.

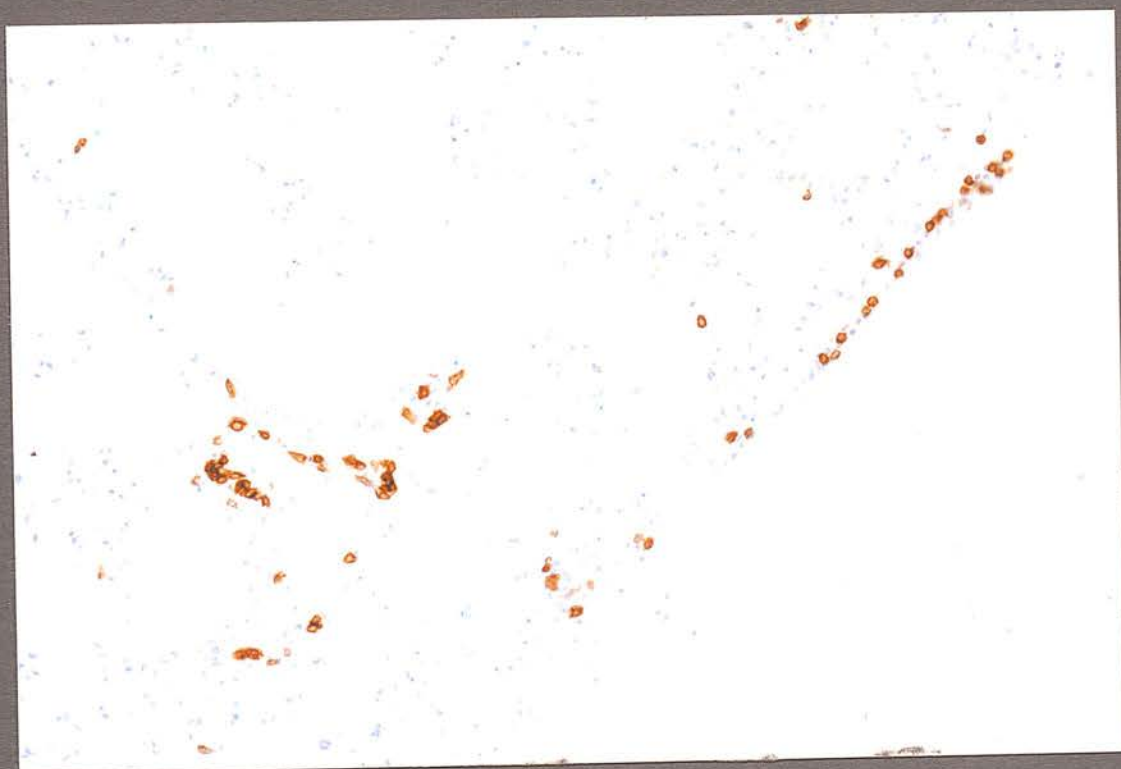
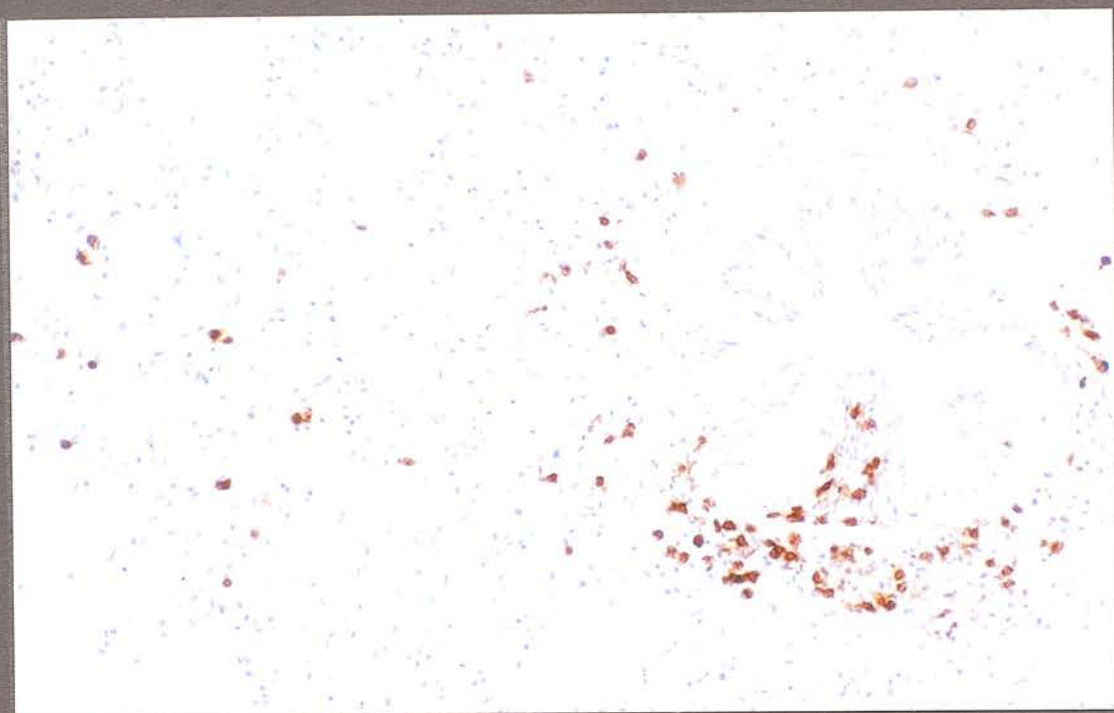


Figure 5.15. $\gamma\delta$ T cells immunolabelled with MAb CC15 in microscopic OPA tumour. Positively immunolabelled cells were detected in the stroma of the tumour and around the periphery. No positive cells were identified in the centre of the tumour. IHC and haematoxylin counterstain. X 250.

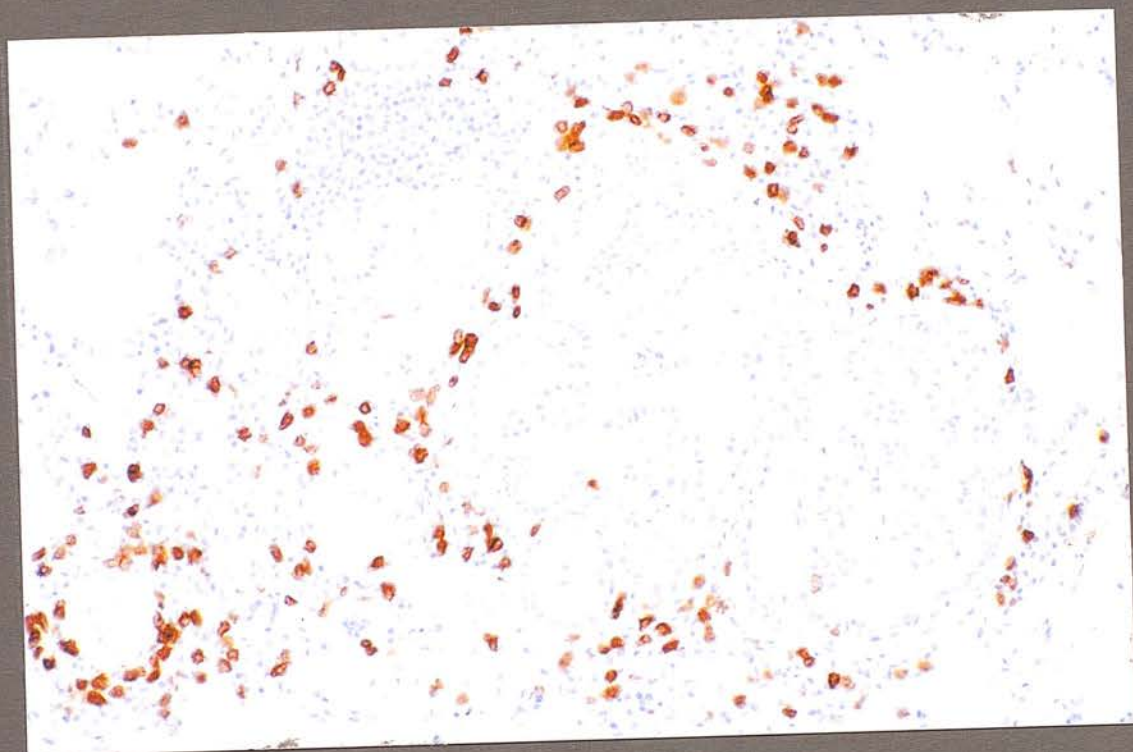


Figure 5.16. Dendritic cells immunolabelled with MAb CC20 in the uninfected lung from a control lamb. The few positively immunolabelled cells that were detected were found in the BALT. IHC and haematoxylin counterstain. X 400.

Figure 5.17. Dendritic cells immunolabelled with MAb CC20 in the non-neoplastic region of OPA lung. Very few positively immunolabelled cells were detected in the alveolar lumen. IHC and haematoxylin counterstain. X 400.

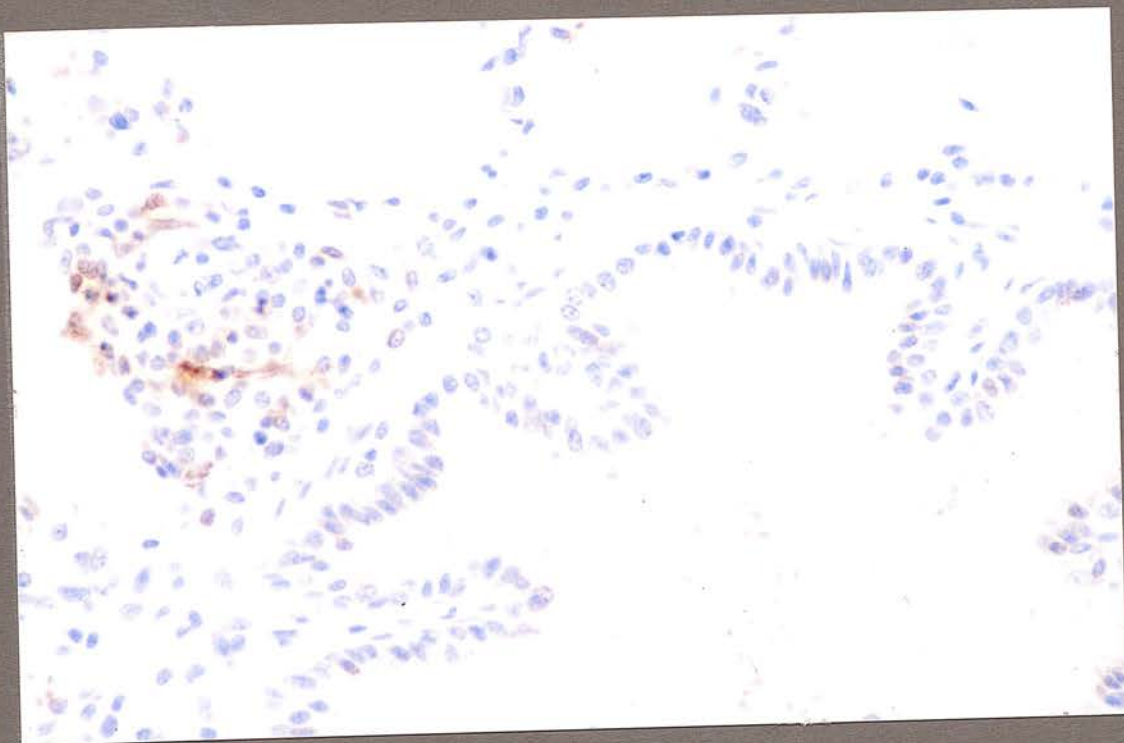


Figure 5.18. Dendritic cells immunolabelled with MAb CC20 in OPA tumour. Very few positively immunolabelled cells were detected at the site of the tumour, with only a few cells found in the periphery. IHC and haematoxylin counterstain. X 400.

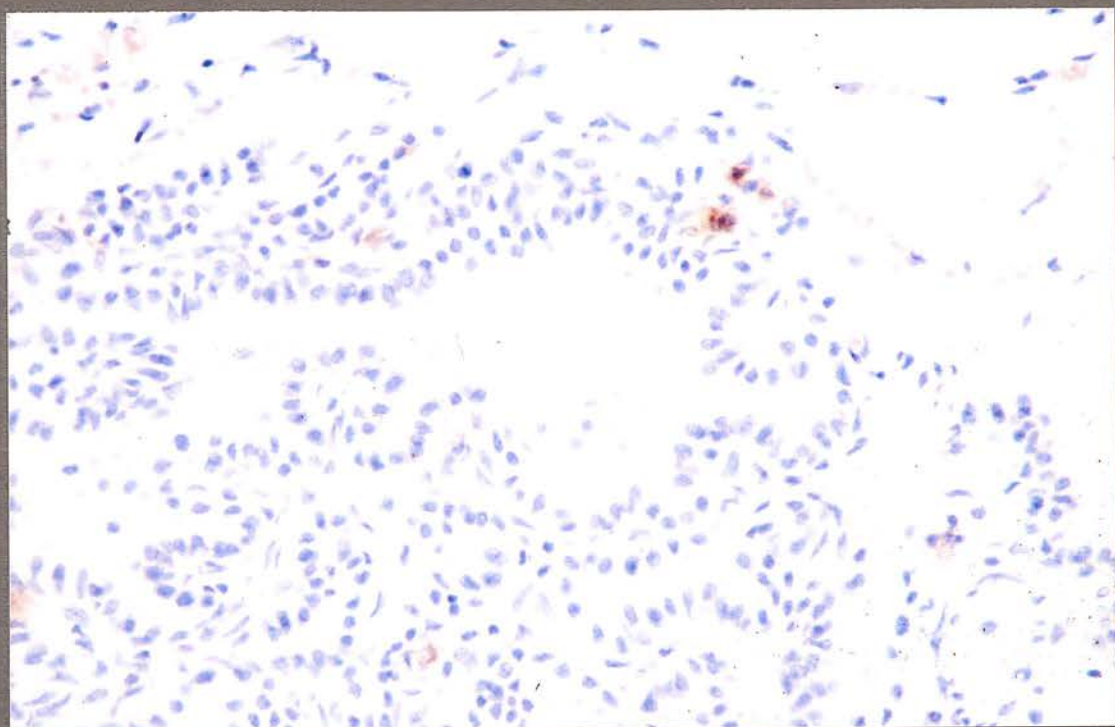


Figure 5.19. Mature B cells immunolabelled with MAb CC21 in the uninfected lung from a control lamb. Positively immunolabelled cells were detected in the BALT. IHC and haematoxylin counterstain. X 100.

Figure 5.20. Mature B cells immunolabelled with MAb CC21 in the non-neoplastic region of the OPA lung. Positively immunolabelled cells were detected in the alveolar walls. IHC and haematoxylin counterstain. X 400.

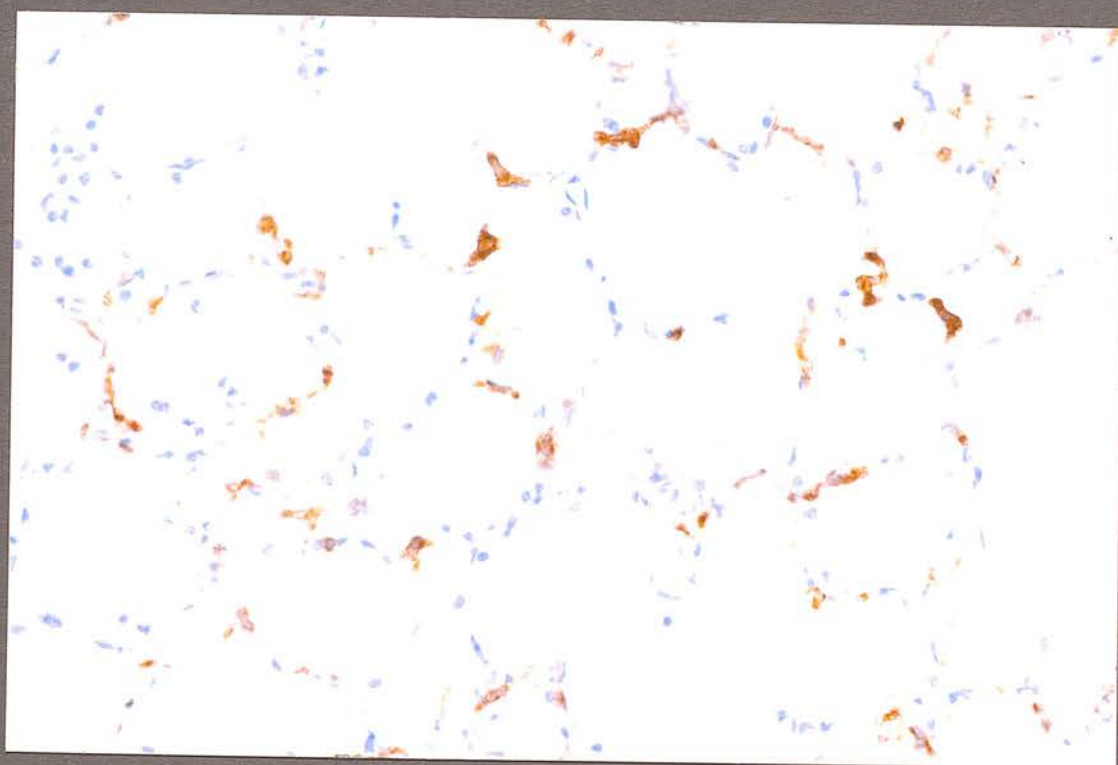
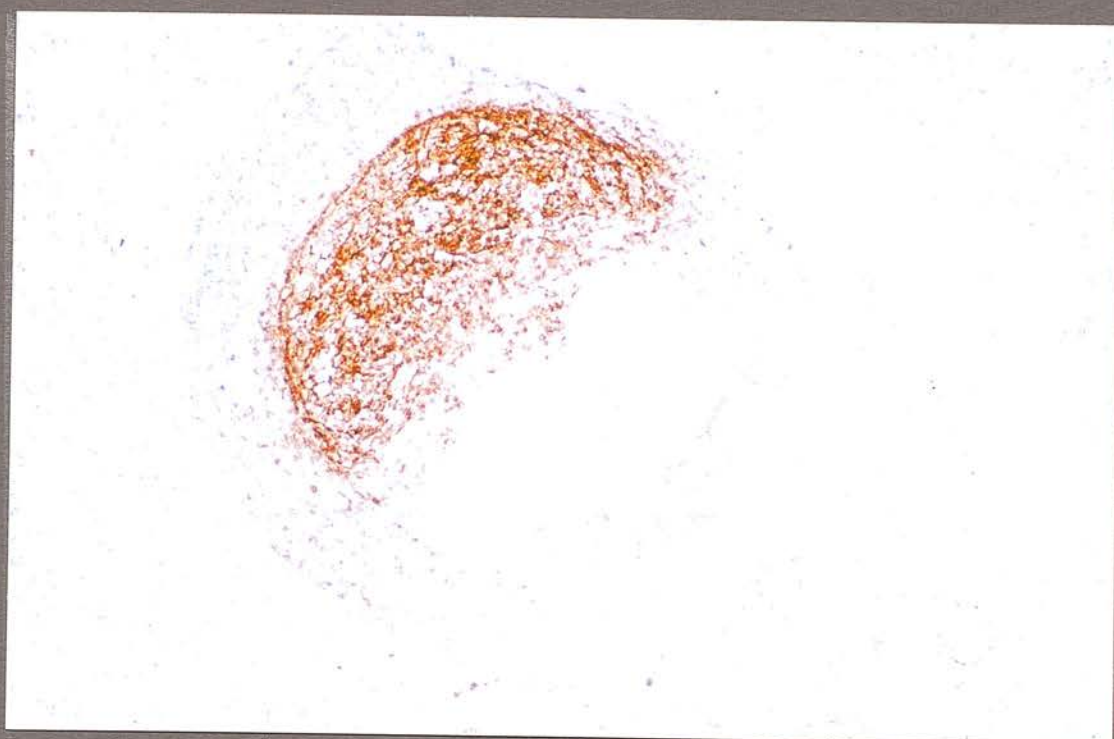


Figure 5.21. Mature B cells immunolabelled with MAb CC21 in OPA tumour. No positively immunolabelled cells were detected in association with the tumour. The positively labelled cells were found only in the parenchyma of the lung. IHC and haematoxylin counterstain. X 400.

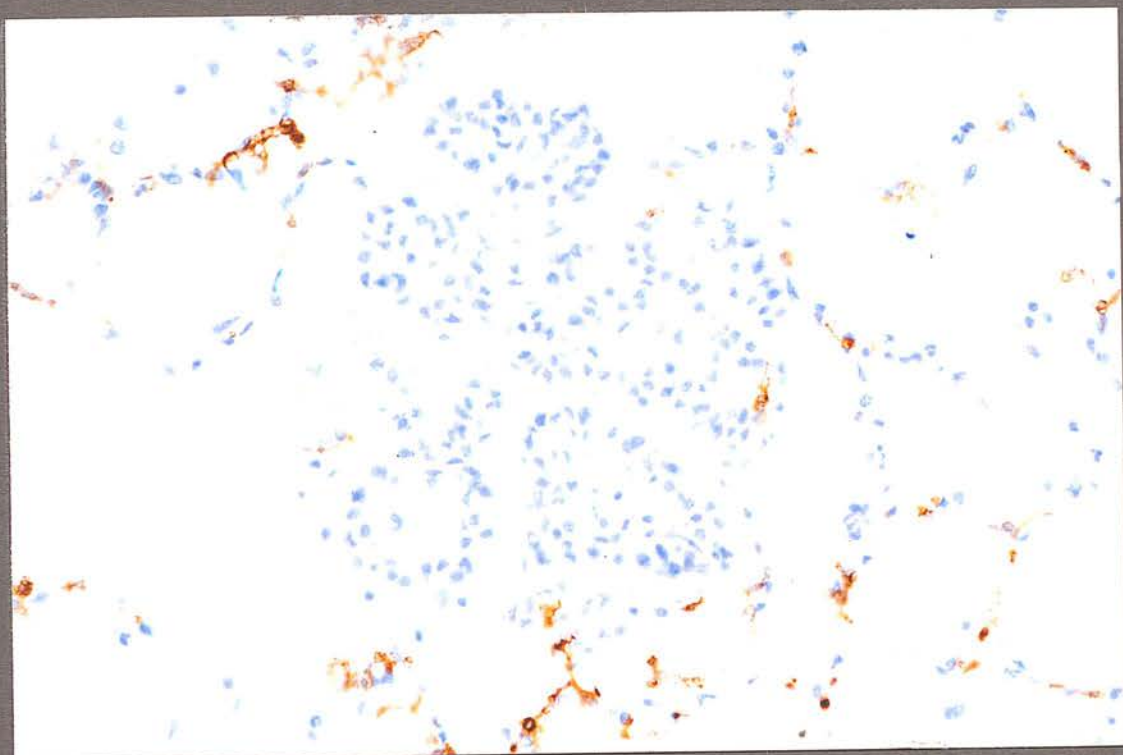


Figure 5.22. Macrophages immunolabelled with MAb VPM65 in the uninfected lung from a control lamb. Positively immunolabelled cells were detected in the alveolar lumen. IHC and haematoxylin counterstain. X 400.

Figure 5.23. Macrophages immunolabelled with MAb VPM65 in the non-neoplastic region of the OPA lung. Macrophages were detected in the OPA lung and appeared larger than those identified in the uninfected lung. IHC and haematoxylin counterstain. X 650.

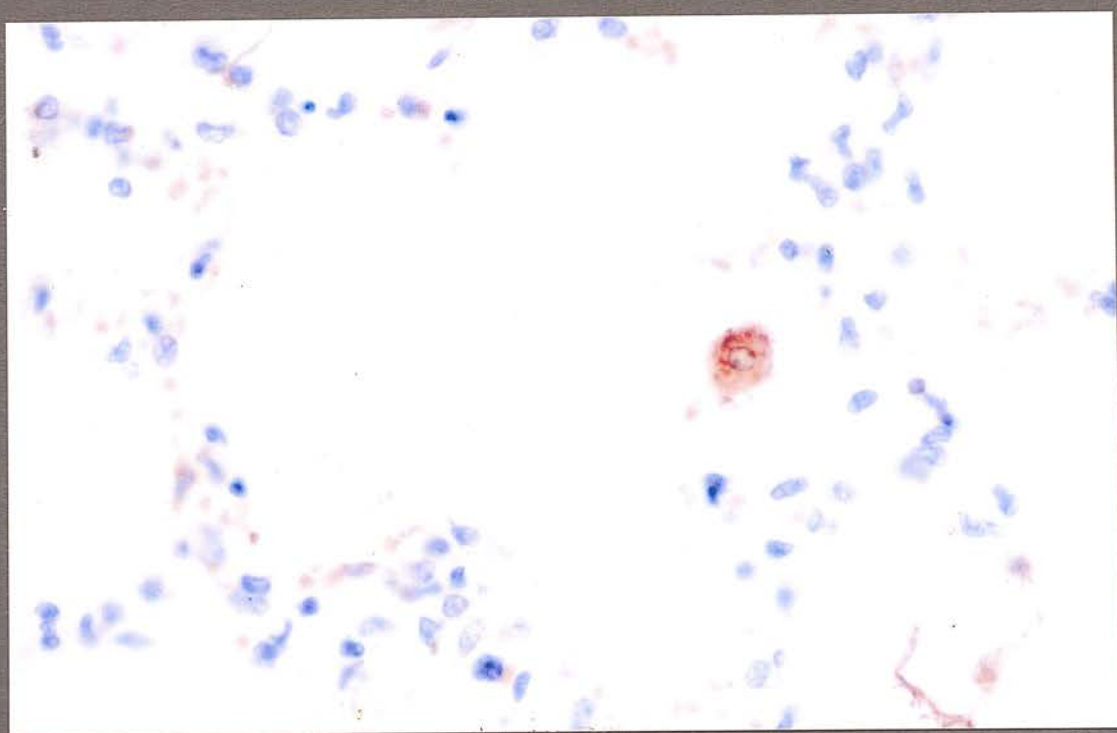
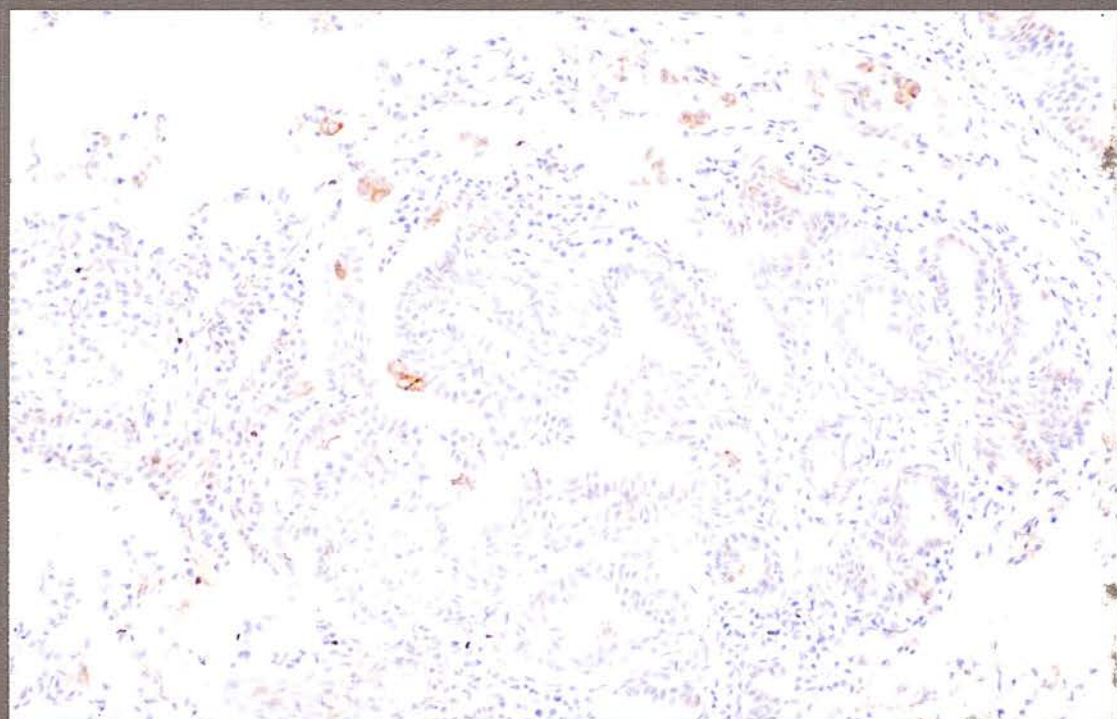


Figure 5.24. Macrophages immunolabelled with MAb VPM65 in the OPA tumour. Positively immunolabelled cells were detected in the stroma and lumen of the tumour. IHC and haematoxylin counterstain. X 250.



5.5 Discussion

This is the first time that ZSF, developed and adapted from the original method reported by Beckstead (1994), has been used to preserve ovine tissues for subsequent immunohistochemistry. The use of ZSF, compared with the traditional methods of aldehyde-fixation or cryopreservation, resulted in establishing an acceptable compromise between retaining cellular morphology, tissue architecture and the preservation of sensitive cell surface antigens, without the need for antigen retrieval. It has been shown that the tissue sections were morphologically almost comparable with aldehyde-fixed tissues and the survival of the antigens was at least comparable, and in some instances superior to cryopreserved.

This study has demonstrated positive immunolabelling of extremely sensitive cell surface epitopes such as CD1, CD4 and CD8, not easily attained in tissues fixed by conventional methods. Furthermore, ZSF resulted in enhanced immunolabelling of more resilient antigens. ZSF can be incorporated into routine, already established procedures of tissue collection, paraffin wax embedding and tissue section cutting, as no extra equipment is required. As the array of monoclonal antibodies continues to increase, and other recognized *in situ* procedures may benefit from this fixation method, the use of ZSF as a primary method of preserving ovine tissues may become more widespread.

Immunolabelling within non-neoplastic regions of the lung from SPF lambs with experimentally induced OPA has shown that, overall, there was very little difference in comparison with uninfected lung tissue from identically housed control lambs. There was no evidence to suggest enlargement of the BALT in the OPA lung and furthermore, the distribution of the lymphocyte subsets throughout the

parenchyma was comparable. No positively immunolabelled B cells, detected by the CC21 monoclonal antibody, were identified either infiltrating the tumour, in the periphery or in the surrounding stroma, but were detected in the parenchyma and the BALT. Although it should be noted that the CC21 monoclonal antibody also recognizes follicular dendritic cells and some of the cells detected in the BALT appear morphology similar to as those detected in the ovine lymph node suggesting that they may be dendritic cells rather than B cells. Unfortunately immunolabelling with the monoclonal antibody VPM8, which recognizes immunoglobulin anti-light chain, was not successful in lung tissue on this occasion. Consequently immunolabelling of B cells in the lung requires to be repeated.

Previous studies have identified lymphocytes in OPA tumour (Dualde-Perez 1966, Hod 1977, De las Heras 1995, Garcia-Goti 2000) and also inflammatory infiltrates have been detected, with numerous neutrophils and enlarged macrophages identified within the lumen (Nobel *et al* 1971, Perk 1982, Sharp and Angus 1985, 1990 and DeMartini 1997). In these studies, tissue from either conventionally housed lambs and adult OPA cases were examined; the use of SPF lambs in this present study may have contributed to the overall lack of tumour infiltration, with the majority of positively immunolabelled cells localised to the periphery of the tumour and the surrounding stroma. However in this investigation, the major difference in the OPA lung, in comparison with the uninfected lung was the presence of a large population of macrophages detected in the BALT and found in large numbers around the tumour nodules. Positively immunolabelled cells were identified within the lumen of the tumour and in close proximity to the tumour epithelium. Although the detection of neutrophils was not part of the present study, an influx of

polymorphonuclear cells, morphologically characterized as neutrophils by microscopic observation, was detected in the region of the tumour from one of the three OPA cases, with macroscopic lesions. In contrast, there was no evidence of neutrophils in the case with microscopic lesions, or in the tumour section from the lamb killed at nine weeks old.

As part of an evaluation of phenotypic frequency alterations during naturally transmitted and experimentally induced OPA, Rosadio and Sharp (1992) reported that a 34-fold increase in the influx of immune cells was detected in the bronchoalveolar lavage (BAL) from three conventionally housed, experimentally infected lambs, compared with two age matched uninfected controls (all lambs 5-6 weeks old). As with the previously mentioned studies, the increase in immune cells was predominantly characterized by alveolar macrophages and neutrophils.

Furthermore in the Rosadio and Sharp study the increase detected in the BAL was not as a result of an alteration in the proportion of T and B lymphocytes but was associated with the proportion of CD4 and CD8 T cells (controls: CD4 56%, CD8 22%, infected lambs: CD4: 41%, CD8 19%). In the present study it would appear that the size (maturity) of the tumour nodule might be influential as CD4 T cells were detected in the region of large tumours, that could be visualised macroscopically and not in small tumours, and CD8 T cells were identified only in the microscopic tumours and not in large nodules. In contrast, the presence of $\gamma\delta$ T cells was not determined by the size of the tumour, as they were detected around all tumour nodules and surrounding stroma, connective tissue and parenchyma. As $\gamma\delta$ T cells are the predominant T cell subset in young lambs and have a specific association with epithelial surfaces, although the localization of $\gamma\delta$ T cells is more prevalent in the

upper respiratory tract than in the bronchoalveolar region of the lungs (Hein and Mackay 1991) the presence of a large $\gamma\delta$ T cell population was not unexpected. In adult field cases OPA pathology has often been accompanied by secondary infections, therefore the exudation of macrophages and neutrophils as a direct response to OPA or as a result of secondary infection remained unclear. However the presence of a large macrophage population and influx of neutrophils has also been detected in conventionally housed lambs with experimentally induced OPA (Rosadio and Sharp 1992, De Las Heras *et al* 1995). An increased population of alveolar macrophages in the region of the OPA tumours, and the detection of neutrophils in one OPA case was detected in the present study, despite reducing the threat of concurrent infections by the use of SPF lambs. Consequently these findings are in accordance with the previous reports that the exudation of macrophages are a property of OPA and do not arise from secondary infection (Dualde-Perez 1966, Hod 1977, De las Heras 1995, Garcia-Goti 2000). However, as tissue samples from only three SPF, OPA lambs were examined in this study, the results presented here are preliminary and further work is required to verify these observations.

To determine if the presence of particular lymphocyte subsets are dependent on tumour size, and to fully analyse any differences in the OPA lung and tumour nodules, compared to uninfected lung a quantitative or semi-quantitative system is required. There are several methods to quantify the results, for example subjective grading of immune cell infiltrates, a system based on tumour measurement and the numbers of positively labelled cells counted within a predetermined area, or utilizing image analysis to quantifying the overall density of immunolabelled cells as described by Johnson *et al* (2000). Johnson *et al* have developed a semi-quantitative

method based on the distribution pattern of immune cells within the infected lung, with an assessment of the degree to which the immunolabelled cells are located either as intratumoural (within the tumour) or peritumoural (within the tumour stroma). This system would not be applicable to this particular study as little evidence of intratumoural infiltration was detected, but may be valuable in quantifying differences within the local draining lymph nodes and for quantitative analysis of tissues from adult OPA cases and uninfected sheep.

The further development of tissue fixation and the expansion of ovine immunohistochemical reagents to study OPA *in situ* would permit future research of immune responses in the microenvironment of the lung, tumour and lymph nodes. JSRV, as the aetiological agent OPA is unique amongst retroviruses as it transforms lung epithelial cells. The incidence of human bronchioloalveolar carcinoma (BAC), a lung cancer only weakly related to smoking and affecting both men and woman is increasing (Wingo *et al* 1998). Macroscopic, histopathological and ultrastructural similarities between OPA and human (BAC) have been demonstrated (Perk and Hod 1982). Consequently an increased understanding of JSRV oncogenesis and OPA development may provide an experimental model for research into human BAC, providing a greater insight into not only ovine but also human pulmonary adenocarcinoma.

Chapter 6
General Discussion
&
Future Work

6.1 Discussion of Results

This investigation, following experimental JSRV infection and in the terminal stages of OPA, has provided evidence for an alteration in cell-mediated immunological response in the peripheral blood of JSRV-infected animals. This alteration was identified as a significantly reduced lymphoproliferative response to ConA stimulation in the PBMC from the blood of conventionally housed and SPF lambs experimentally infected with JSRV, and also in adult sheep in the terminal stages of OPA. In contrast, no difference between the JSRV-infected and control animals was detected when the PBMC were stimulated with PHA, PWM, or NPA. NPA proved to be mitogenic for the PBMC from lambs and not adult sheep. Although no difference in the level of lymphoproliferation following NPA stimulation was detected between JSRV-infected and control lambs, evidence that the mitogenicity of this lectin was affected by the presence of the virus was demonstrated by an alteration in the post-stimulation T and B cell profiles identified between the infected and control lambs. Further evidence to substantiate an altered cellular response in the JSRV-infected lambs was demonstrated when only partial inhibition of NPA mitogenicity could be induced by the addition of exogenous mannose, in contrast to complete NPA inhibition detected in age-matched control lambs. These preliminary findings may suggest (amongst the other possibilities described in Chapter 4) that lymphoproliferation to NPA stimulation was induced *via* a different pathway by the presence of JSRV.

Further investigation of the ovine response to stimulation by ConA and NPA revealed that for both mitogens, proliferation of CD4 T cells was detected, with only a limited response in $\gamma\delta$ T cells. The results also suggested that ConA and NPA

stimulation induced B cell proliferation that is possibly T cell dependant. In contrast however, the mitogenicity of the two mitogens differed in that mitotic division of CD8 T cells was induced by ConA but not by NPA stimulation. Possibly the virus affects the surface molecules of CD8 T cells, which could explain the reduced response detected following ConA stimulation and not to NPA stimulation in JSRV-infected lambs.

The requirement for antigen presentation in the mitogenicity of the lectins, determined by the depletion of adherent cells from the PBMC, demonstrated that accessory cells were required for NPA mitogenicity. Furthermore, it was demonstrated that the level of lymphoproliferation to ConA stimulation was influenced by the presence of antigen-presenting-adherent cells to a greater extent than with PHA or PWM. A study by Larsson *et al* (1980) demonstrated that ConA can bind directly to the lymphocyte membrane inducing IL-2 expression, but this alone was not found to be sufficient to induce lymphoproliferation and that other signals were required. This was elucidated when Klaus and Hawrylowicz (1984) identified that a second signal from accessory cells was necessary for T cell synthesis and expression of the IL-2 receptor. The requirement of accessory cells for the mitogenicity of some mitogens was first suggested by Habu and Raff (1977) and since then many studies have attempted to ascertain whether the whole accessory cell or soluble factors alone are sufficient. A series of experiments by Gallagher *et al* (1986) demonstrated that soluble factors, derived from activated accessory cells were not sufficient to induce lymphoproliferation to ConA stimulation. Similar studies performed by others are in accordance with these findings, whilst other groups demonstrated conflicting evidence. For example Maizel *et al* (1981) and Palacios

(1982) found that lymphoproliferation to ConA in the presence of soluble factors alone fully substituted for the intact cells. It was reported in the Gallagher *et al* (1986) study that the physical presence of accessory cells was required to promote aggregation of the cells in culture. It was demonstrated however that depletion of accessory cells did not result in complete loss of cross-linking. Consequently, it was concluded by Gallagher *et al* that the requirement of accessory cells in ConA mitogenicity was not absolute, which is in accordance with results presented in this present study.

Phenotypic alterations demonstrated in the peripheral blood were confined to the terminal stages of OPA, and no detectable level of IFN- γ , specific to infected animals, was detected throughout the experimental infections. Consequently during experimental JSRV infection, there was no evidence to suggest that an immunological response involving IFN- γ was induced. The reason why JSRV appears to be undetected by the immune system is still unknown but several possibilities can be considered.

6.2 Tolerance to JSRV antigens

It has been established that the sheep genome contains copies of endogenous retroviral sequences highly related to the exogenous form of JSRV (detailed in Chapter 1, section 1.5.1). Several studies by Palmarini *et al* (1996a, 2000a, 2000b, 2001) revealed that endogenous JSRV antigens, closely related to exogenous sequences are transcriptionally active in a wide range of tissues. It was demonstrated that the endogenous and exogenous forms of JSRV have different tissue activity, with expression controlled by the viral LTR. The exogenous JSRV has specificity for

the type II pneumocytes in the lung. In contrast, endogenous JSRV sequences were found in the gut and also in the uterus. Furthermore it was shown that the level of endogenous RNA expression in the uterus was related to hormonal levels, oestrous cycle and early pregnancy. It was suggested also that the endogenous JSRV might play a role in placental morphogenesis. Consequently expression of these sequences during foetal ontogeny could lead to the clonal deletion or clonal anergy of JSRV-specific T and/or B lymphocytes, thereby establishing a state of tolerance for the endogenous form of the virus. The immune system, subsequently encountering exogenous JSRV would not identify an infection as the exogenous antigens would be regarded as self. Consequently no immunological response would be induced. Studies have shown that mice carrying endogenous Moloney murine leukaemia virus (M-MuLV) do not develop antibodies or cytotoxic T lymphocyte (CTL) responses to the antigenically cross-reactive Murine sarcoma virus (MSV) (Ronchese *et al* 1984). Furthermore, mice infected with M-MuLV have a lower number of CTL precursor cells suggesting clonal depletion of virus specific T cells. Mouse mammary tumour virus (MMTV), with some homology with JSRV can also exist in two forms, either as a transmissible virus particle or as an endogenous provirus. The life cycle of MMTV depends on eliciting an immune response to promote lymphocyte proliferation for the transportation of the virus to site of tumorigenesis as detailed in Chapter 1, section ???. It has been shown that mice carrying the endogenous MMTV have reduced superantigen (Sag)-specific CTL responses, and that temporally the Sag-specific T cells are depleted from the T cell repertoire, once the disseminated infection has been established (Acha-Orbea *et al* 1999). Further evidence to substantiate a tolerance hypothesis for JSRV is the lack of JSRV-specific circulating

antibodies in naturally infected adult OPA cases and during experimental JSRV infection (Sharp and Herring 1983, Verwoerd *et al* 1990, Ortin *et al* 1998, van der Molen personal communication). Clonal deletion of immune cells specific for transcriptionally active endogenous retroviral antigens encountered during foetal development can therefore be augmented by peripheral tolerance or clonal anergy of cells specific for antigens not previously encountered *in utero*. There is no evidence to suggest that cells specific for JSRV are deleted from the system but rather that they had been rendered immunologically nonresponsive. It has been shown that JSRV-specific antibodies have been detected in sheep immunised with recombinant JSRV capsid protein (Dr. Renate van der Molen unpublished results). Furthermore, in the early stages of experimental JSRV-infection and tumour development, phenotypically in the peripheral blood, there was no evidence supporting the deletion of any particular lymphocyte subset. Subsequently however, during the terminal stages of JSRV infection the demonstrated CD4 lymphocytopaenia could suggest peripheral tolerance by clonal deletion or possibly downregulation of the TcR, the CD4 accessory molecule or both factors.

The preliminary *in vivo* phenotyping of tumours from SPF lambs with histopathologically confirmed OPA detected CD8 T cells only in microscopic tumours that were absent in large tumours, perhaps suggesting their deletion from the microenvironment as neoplasia progressed. However there was no evidence to support this hypothesis in the phenotypic profile of the peripheral blood from these JSRV-infected lambs, nonetheless it could be suggested that early responses elicited in the microenvironment of the lung are undetectable systemically. Alternatively this observation may suggest that cytokine imbalance, the acquisition of additional cell

markers or inhibitory factors may be produced during tumour development that could confer protection from the immune system.

Previous studies examining the role of cytotoxic T lymphocyte (CTL) responses to retroviral infections have shown that, although antibody responses are extremely important in controlling the course of infection and evolution of the virus population, the cell-mediated recognition of infected cells rather than free virus is of equal importance in combating infection. For example, a study by Plata (1985) revealed that Balb/B mice, which are able to reject cells transformed with Gross murine leukaemia virus (GMLV) developed tumour after suppression of the CTL response. Cerundolo *et al* (1987) studying Gambian female prostitutes, who were repeatedly exposed to HIV and remained seronegative, suggested CTL protective immunity as they were found to have CTL responses (Rowland-Jones *et al* 1995). It has been shown also however, that despite high HIV-specific CTLs detected in infected individuals, the infection continues unabated (Wei *et al* 1995). It has also been shown that specific CTLs have been detected in sheep with maedi-visna virus (MVV), but despite this the infection was found to persist (Blacklaw *et al* 1994). Cell-mediated immunity may play an important role during early JSRV infection and tumorigenesis, but possibly the effectiveness of the CTL response is downregulated as neoplasia progresses.

6.3 Immunosuppression

As described in the discussion in Chapter 4, there are several examples of retrovirally-induced immunosuppression of the lymphoproliferative response to mitogen stimulation. Examples include the transmembrane protein p15E of FeLV

(Mitani *et al* 1987) and murine leukaemia virus (MuLV) (Cianciolo *et al* 1990), and also Mason-Pfizer monkey virus (MPMV) (Blaise *et al* 2001). Furthermore immunosuppression can be selective for specific mitogens. Two parallel studies in 1981 by Warner *et al* and Fleisher *et al* examined human T cell proliferation and B cell immunoglobulin (Ig) synthesis following ConA stimulation. It was revealed that a subset of cells differentiated into suppressor cells that synthesised and secreted soluble factors that not only inhibited cell-cell interactions, but also negatively modulated cellular function and humoral immune responses. The first factor, human soluble immune suppressor supernatant (SISS) was either produced directly by adherent cells or required adherent cell involvement. It was demonstrated that this factor inhibited mitogen and antigen-stimulated T cell proliferation (SISS-T). Competitive binding assays demonstrated that SISS-T binds to receptors on the surface of T cells that are also recognised by inhibitory lectins such as wheat germ agglutinin (WGA), suggesting that SISS-T appeared to be an endogenous lectin-like soluble suppressor induced by ConA stimulation. Furthermore it was demonstrated that the inhibitory mechanism of SISS-T did not involve cell death. The second factor identified was SISS-B, found to inhibit B cell immunoglobulin production and was produced by T cells. Characteristic differences were identified between the two soluble suppressors, for example the ability to block SISS-T activity with N-acetyl-D-glucosamine, which did not affect SISS-B; whereas the addition of L-rhamnose did result in blocking SISS-B. Therefore the effect of ConA mitogenicity in the human system has been shown to synthesis two diverse soluble factors, with distinct cell surface receptors, which negatively modulate both the cell-mediated and humoral responses. Perhaps, if analogous suppressors are produced in the ovine

system, the presence of JSRV may modify their synthesis and secretion resulting in immunosuppression and the reduced lymphoproliferative response to ConA stimulation.

Viral modification of post-recognition events, such as transport mechanisms and the intracellular pathways utilized during mitogenesis, have been studied in a range of systems where retroviral proteins have been found to disrupt several signal transduction pathways. For example, the Arg-Arg amino acid residues comprising the N-terminal of the synthetic peptide CKS-17 and the homologous transmembrane peptide derived from HIV-gp41 are involved in the increased production of cAMP (Haraguichi *et al* 1995). Elevated cAMP levels, inhibition of protein kinase C (PKC) activity and influx of intracellular Ca^{2+} ions suppresses activation of PKC-dependent lymphocytes (Ruegg *et al* 1991). The PKC-dependent pathway is utilised by activated $\text{T}_{\text{H}}1$ cells for the production of IL-2, whereas the $\text{T}_{\text{H}}2$ signal transduction mechanism is PKC-independent for the production of cytokines such as IL-4 and cell proliferation (Tamura *et al* 1993). The glycoprotein gp120 of HIV-1 suppresses PHA-induced stimulation by inhibiting signal transduction through the T cell receptors and CD3 complex (TcR/CD3) (Cefai *et al* 1990). This, in conjunction with the larger regulatory complex involving tyrosine kinases and phosphatases, results in the reduced responses to PHA stimulation (Mustelin and Altman 1991, Haugan *et al* 1992). Despite strong evidence of the immunosuppressive potential of HIV-gp120, there is little indication of such activities for SU-proteins from other retroviruses, thus far. Although these specific mechanisms may not relate directly to JSRV, viral modification of post-recognition events utilised during mitogenesis may be involved. Recent studies have identified that within the transmembrane subunit of the JSRV

envelope, the cytoplasmic moiety is sufficient for the transformation of NIH-3T3 cells. Furthermore phosphorylation of protein kinase B is detected in all JSRV-transformed cells, indicating that expression of the JSRV envelope results in activation of the phosphatidylinositol 3-kinase (PI-3K) cellular signalling pathway (Palmarini *et al* 2001). This pathway, induced by the phosphorylation of protein kinase B by PI-3K is known to inhibit apoptotic mechanisms and is essential for the transition from G1-to-S-phase during the lymphoproliferative response to several mitogens (Roche *et al* 1999). Signal transduction and regulatory mechanisms may be influential, not only in contributing to the neoplastic process but also in determining the altered cellular immune response of JSRV-infected sheep.

6.4 Evasion of the immune system

As many viruses use survival strategies to evade immune detection, it should be considered that JSRV may also utilize a method to subvert exposure to the immune system. Many studies to identify virus survival mechanisms have examined the methods employed by the large DNA viruses, which carry a large complement of specialised genes that are not found in retroviruses. Notwithstanding, this provides an insight into the multitude of evasion tactics used. To be recognised by the immune system viral antigens must be presented, in conjunction with major histocompatibility complex (MHC) on the cell surface, (the process of antigen presentation by infected cells expressing MHC class I and class II is described in Chapter 1, section 1.3.4). Consequently many survival strategies have evolved which prevent antigen presentation on the cell surface. The simplest method is failure of the viral protein to be degraded by the proteosomes. This is the mechanism, identified by

Levitskaya *et al* (1995), utilized by Epstein-Barr virus (EBV), where the protein EBNA-1 from EBV-infected B cells is not processed by the proteosomes because its amino acid structure contains Gly-Ala repeats, which cannot be processed by the proteolytic enzymes. If a protein is processed to create small peptides then interference with peptide uptake from the cytosol by the TAP proteins inhibits transportation to the MHC molecule. Herpes simplex virus (HSV) expresses an immediate early protein ICP47, which on binding to TAP prevents the translocation of the peptide to the ER (Hill *et al* 1995). If MHC can be prevented from reaching the cell surface then antigen presentation cannot occur. Accordingly there are many evasion techniques to retain MHC within the cell. MHC can be retained in the ER, a strategy adopted by certain strains of adenovirus, the viral proteins having a high affinity for MHC and also the ability to hold the protein-protein complex within the ER by the presence of an ER retention signal in the cytoplasmic tail of the adenovirus protein (Jackson *et al* 1990, Cox *et al* 1991). Furthermore an adenovirus coat protein, on entry into the *cis*-Golgi mediates retrograde transportation back into the ER (Jackson *et al* 1993). If an antigen-MHC class I complex should form it requires to travel through the ER and Golgi apparatus to reach the surface of the cell. The murine cytomegalovirus (MuCMV) m152 protein retains the MHC/antigen trimolecular complex in the ER-Golgi intermediate compartment (Del Val *et al* 1989, Ziegler *et al* 1997), whereas human cytomegalovirus (HuCMV) encodes an ER resident glycoprotein that relocates the newly synthesised MHC class I molecule to the cytosol where it is subjected to proteolysis (Wiertz *et al* 1996a, 1996b). It has been demonstrated that cells devoid of, or expressing low levels of MHC class I are susceptible to destruction by natural killer (NK) cells (Ljunggren and Karre 1990).

Therefore, in order avoid NK cell detection HCMV has extra open reading frames m144 and UL18, which encode proteins homologous to MHC class I proteins. Expression of these viral proteins mimic MHC class I expression and subvert NK cell mediated lysis (Reyburn *et al* 1997).

Other methods of evading immune detection have evolved with viral production of proteins, which mimic immunomodulatory molecules. For example HIV-type I, EBV and adenoviruses inhibit activation of the protein kinase pathway by producing small RNAs that bind to the protein kinase receptor (PKR) (Imani and Jacobs 1988, Mathews and Shenk 1991, Levitskaya *et al* 1995). Vaccinia virus also produces a soluble protein, which is a pseudo-substrate for PKR, inhibiting functional activation and furthermore produces a soluble protein that binds the pro-inflammatory cytokine IL-1 β (Akkaraju *et al* 1989). Several viruses produce soluble molecules homologous to cytokine receptors, which intercede and prevent them from reaching their target cells. Herpesvirus *saimiri* encodes a molecule similar to the human IL-8 receptor, therefore inhibiting the activity of the chemoattractant (Albrecht *et al* 1992). IL-6 can be bound by a protein from hepatitis B virus (Chisari and Ferrari 1995), and EBV encodes a homologue of IL-10, which inhibits IFN- γ (Moore *et al* 1990). Adenovirus uses a different strategy by encoding three proteins, which inhibit TNF induced lysis of the infected cell (Gooding *et al* 1988). There is no evidence thus far to suggest that JSRV evades immune detection by subversion as no accessory proteins are encoded, however the product of *ORF X* remains to be identified. Further investigation of the relationship between JSRV and the type II pneumocytes, which themselves have immunomodulatory functions, may identify

virus induced alterations in, for example MHC class I or class II expression or a cytokine balance within the lung that would be beneficial to JSRV survival.

6.5 Future Work

The mechanisms of mitogen mitogenicity: Further research is required to determine the mechanism(s) of ConA and NPA mitogenicity for sheep lymphocytes. It should be determined if NPA mitogenicity is induced *via* the mannose pathway and/or alternative signal transduction pathways that may be utilised in the responses from neonatal, control and older JSRV-infected lambs. The data generated may provide an explanation for the altered cellular response to mannose inhibition of NPA mitogenicity detected in the JSRV-infected lambs. An investigation of interactions between the lectins and the lymphocytes, at the cell surface and intracellularly should be performed to elucidate the altered cellular response to ConA stimulation during JSRV infection.

As IFN- γ was the only cytokine measured during this study, other factors should be monitored in the blood and also cytokine release during ConA and NPA stimulation, for example IFN- γ , GM-CSF, IL-2, IL-4 or IL-10 to determine the type of effector response induced, and identify any cytokine alterations detected during JSRV infection. Studies to determine if ConA and NPA induce the synthesis of ovine suppressor factors, which may be altered by the presence of JSRV, should be included.

The influence of antigen presentation: Due to insufficient time, and the availability of lambs only in the spring and autumn months, it was not possible to extend the findings of this study. Therefore, this work should be expanded to fully investigate

the requirement for antigen presentation in the mitogenicity of ConA and NPA. Studies of the direct physical association of antigen presenting cells on the lymphoproliferative response, and any involvement of soluble mediators should be established to analyse the role of antigen presenting cells in instigating mitogenicity. Furthermore, the requirement for accessory cells in correlation with the demonstrated age-related mitogenicity of NPA should be considered. The lymphoproliferative responses were only tested in uninfected control lambs during this study. Therefore this should be extended to compare uninfected and JSRV-infected animals.

The development of anti-viral T cell responses is dependent on interactions between antigen presenting cells and naïve T cells. Although dendritic cells (DC) are regarded as the professional antigen presenting cells (APC), the important role of monocytes/macrophages as APC should be examined in JSRV infection. As the disseminated infection identified with JSRV, demonstrated that the highest proviral load was found in the monocyte/macrophages lineage (Holland *et al* 1999) studies should be undertaken to determine any association with antigen presentation in the altered cellular responses detected during JSRV infection. For example, it has been reported that HCMV infection of macrophages elicits changes in the cell, such as disruption of the microtubule network resulting in vacuole formation, and a downregulation of MHC class II molecules on the cell surface, which may support intracellular persistence and immune evasion. Furthermore HCMV infection downregulates gene expression of the lipopolysaccharide (LPS) receptor CD14, presumably increasing susceptibility to concurrent bacterial infections in the lung (Hengal *et al* 1998). Further investigation into the effects of JSRV on dendritic cells (DC) is required also to ascertain if the virus can infect the professional antigen

presenting cells, and if so, determine whether JSRV induces alterations in their effector functions that could interfere with the development of specific immune responses. Studies of DNA and RNA viruses have demonstrated that the DC are potent inducers of CTL responses and stimulate the proliferation of naïve T cells and the secretion of cytokines. It would be advantageous for a virus to infect and impair the functions of DC. It has been demonstrated that measles virus (Schneider-Schaulies and ter Meulen 1999, Server-Delprat *et al* 2000), HIV (Rubbert *et al* 1998) and HSV-1 (Kruse *et al* 2000) infect DCs. A study by Sevilla *et al* (2000) demonstrated that the receptor on DC and the interaction with lymphocytic choriomeningitis virus (LCMV) virus was a crucial stage in LCMV-induced immunosuppression and viral persistence.

Measurement of cellular and antibody responses: At the present time adult sheep have been immunised with a recombinant JSRV-capsid antigen (CA)/GST construct and boosted with recombinant JSRV-CA/ β gal and JSRV-CA/GST. A programme to measure JSRV-specific cellular immune responses and detect JSRV-specific antibodies in the peripheral blood is currently underway (Dr. Renate van der Molen, personal communication).

Immunohistochemical analysis of the OPA lung and lymph nodes: Further research is required to extend the preliminary findings of this study. *In situ* phenotyping of a larger number of samples should be undertaken, with serial sections of tumour and non-neoplastic lung phenotyped and quantitatively analysed. Comparative analysis of tissues from lambs and adult OPA cases would provide information regarding any differences in tumour infiltration arising from experimental and natural JSRV infection and also age-related differences in the lung. If alterations induced by JSRV

infection are not detectable in the peripheral blood then *in situ* assays to detect and measure intracellular cytokine expression and soluble mediators need to be developed. Unfortunately due to time restrictions immunochemical analysis of the pulmonary lymph nodes was not possible. Comparative *in situ* phenotyping of the mediastinal and bronchial lymph nodes from OPA and control lambs should be performed, again using serial sections to identify any alterations in the profiles of immune cells and cellular morphology. Moreover the lymph nodes from experimentally infected lambs and adult OPA sheep should be compared to determine any age-related differences. Several factors such as viral persistence and age at infection are important in determining the mechanisms of virus-induced immunosuppression (Tishon *et al* 1993, Ahmed and Oldstone 1988, Evans *et al* 1994).

Investigation of a possible mechanism to transport JSRV to the lower respiratory tract: During experimental infection JSRV is administered intratracheally, resulting in direct inoculation of the lower respiratory tract by a high dose of virus. In contrast during naturally transmitted JSRV infection the suspected mode of transmission is inhalation of aerosols from infected flock mates. Therefore as JSRV dissemination was shown to be an early event preceding tumorigenesis (Holland *et al* 1999), studies should be extended to examine the lymphoid tissues in the upper respiratory tract of naturally infected sheep. As JSRV has some homology with MMTV it would be interesting to determine if JSRV utilizes a viral transport mechanism similar to MMTV to translocate the virus from the upper respiratory tract to the bronchioalveolar region of the lung. Although there has been no evidence to suggest that JSRV encodes a superantigen, the identity of the viral product encoded by the

extra open reading frame *ORF-X* is still unknown. A link between *ORF-X* and JSRV dissemination may be provided by the recent evidence of dissemination during caprine nasal adenocarcinoma virus (CNAV) infection, which possesses the extra open reading frame *ORF-X*. Contrastingly no dissemination was evident during ovine nasal adenocarcinoma virus (CNAV) infection, which does not have the *ORF-X* (C. Cousins, A. Ortin personal communications). This is not to suggest that *ORF-X* encodes a superantigen, but possibly a product involved in viral infection of the lymphatic system. For example, the concept that the herpes virus EBV is transmitted in saliva and establishes a latent infection in circulating B cells. However viral DNA has been detected in epithelial cells in oral hairy leukoplakia (OHL) and also in nasopharyngeal carcinoma suggesting the possible transfer of virus from B cells to epithelial cells. There is no evidence that EBV encodes a superantigen to instigate translocation. However, the upper respiratory tract contains aggregates of lymphoid tissues, including the nasopharyngeal lymph nodes and the pharyngeal tonsils. The tonsils are composed of two different forms of tonsillar tissue, the surface, stratified squamous epithelium, invaginated into the underlying connective tissue to form crypts. The reticular epithelium within the crypts is loosely formed with disruptions, through which lymphoid cells infiltrate to form lymphoepithelium (Faulkner *et al* 2000). The crypts therefore provide a first line of defence against infection of the respiratory tract. The mechanism postulated by Faulkner *et al* (2000) for EBV crossing the epithelial barrier is by transcytosis, an infection independent vesicular transport system that has also been demonstrated *in vitro* for HIV (Bomse 1997). It has also been shown that HIV exploits M cells as a transport mechanism from the crypt lumen to the lymphoid tissues, for subsequent infection of macrophages and

CD4 T cells (Neutra *et al* 1996). Stahl-Henning *et al* (1999) demonstrated the presence of infected lymphoepithelium in the tonsils of macaques inoculated with simian immunodeficiency virus (SIV). Furthermore the spread of SIV to other lymphoid tissues and peripheral blood was subsequently identified. As JSRV dissemination is detected early during infection a possible mechanism of viral transportation to the lung should be investigated.

NPA mitogenicity for human lymphocytes: This preliminary study has provided a foundation for further research into the mitogenicity of NPA for human subjects. The lymphoproliferative responses of other age groups, with increased sample numbers, should be analysed to determine the mitogenicity of NPA for all generations. The identification of human lymphocyte subsets responding to NPA, the requirement for antigen presentation, cytokine expression and mannose inhibition of NPA mitogenicity (as performed in this project, and detailed in this section, for sheep) should be investigated in humans. Once the mechanisms of NPA mitogenicity and effects on lymphocytes from healthy subjects are fully established, then any alterations detected during infection could be investigated. NPA therefore, as an agent in the fight against retrovirus infection, as detailed in the Introduction to Chapter 4, may have potential parallel applications for research into human and sheep bronchioloalveolar carcinoma.

6.6 Concluding statement

This study has shown overall, with the notable exception of JSRV induced alterations in cellular function, during experimental JSRV infection and tumorigenesis that there was no evidence, systemically, to distinguish infected from

uninfected lambs. The CD4⁺ lymphocytopaenia and neutrophilia detected in the blood of adult sheep during the terminal stages of OPA as reported by Rosadio and Sharp (1992) and Holland *et al* (1999) were also identified in this study, confirming that these events occur post-neoplasia and are therefore confined to the terminal stages of OPA. It would appear from the existing evidence and from the data generated during this project that JSRV is relatively immunologically silent. Therefore, future studies as described and investigation into possible modes of infection, such as vertical transmission and the detection of virus in the milk are crucial in elucidating the lack of immunological response. Moreover the development of immunological assays to examine the interactions between the virus and the host cells, systemically, in the lymph nodes and in the microenvironment in the lung are required to increase our understanding of JSRV infection and development of OPA.

Chapter 7

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Detection of Immune System Cells in Paraffin Wax-embedded Ovine Tissues

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Summary

This report describes a method (fixation, paraffin wax-embedding and immunolabelling) for the demonstration of several immune system cell epitopes (CD1, CD2, CD4, CD8, CD14, CD21, CD45R, WC-1, 28 kDa surface antigen, immunoglobulins and MHC II antigens) in ovine lymph nodes collected at necropsy. Cell surface epitopes considered to be sensitive to processing methods were successfully demonstrated by a procedure that included the use of a non-aldehyde-containing, zinc salts-based fixative, coupled with a sensitive system of immunolabelling. This novel method had the advantage of avoiding antigen-retrieval steps and of providing consistently good morphological definition.

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Introduction

Studies on the pathogenesis of animal diseases often require the demonstration of pathogen-specific or host-cell-specific antigens. While alternative methods such as fluorescence-activated cell sorting (FACS) or enzyme-linked immunosorbent assay (ELISA) are available to detect and quantify such antigens in tissue samples, immunohistochemistry (IHC) is the method of choice for their “in-situ” demonstration. Unlike the other methods, IHC enables a detailed assessment to be made of the tissue distribution of antigens and of the spatial relationship between the cells that express them and other cells and tissue structures. A prerequisite, however, is good preservation of tissue morphology and antigenicity.

The use of conventional aldehyde-based fixatives (formaldehyde and paraformaldehyde, and their derivatives), and to a lesser extent alcohol-based fixatives, results in good morphology but poor antigen preservation. Aldehyde-based fixatives establish cross-linking bridges between the cells,

and these bridges often mask the cell surface antigens (Fox *et al.*, 1985), which complicates the identification of lymphocytes and other cells of the immune system. To overcome this difficulty, several approaches have been adopted, including a search for antibodies that react with processing-resistant epitopes (Gerdes *et al.*, 1992), as well as modification of the fixation, processing or embedding procedures (Beckstead, 1985; Sato *et al.*, 1986), notably the addition of zinc compounds to aldehyde fixatives (Mugnaini and Dahl, 1983; Tome *et al.*, 1990). The use of post-embedding antigen retrieval steps has also been explored (Cattoretti and Suurmeijer, 1995; Miller *et al.*, 1995), as has pre-embedding labelling of antigen in thick sections obtained with a vibrating microtome (Totterdell *et al.*, 1992). Although most of these procedures have resulted in an improvement of antigen survival, none has been widely adopted, perhaps because they require complicated changes to normal practice or work only in specific instances and are therefore not broadly applicable.

Beckstead (1994) reported a procedure for the detection of fixation-sensitive antigens in paraffin wax-embedded human tissues, based on the use of a non-aldehyde fixative containing zinc salts (zinc

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Table 1
Immunohistochemical demonstration of ovine cell surface antigens by Mabs after ZSF fixation and paraffin wax-embedding of lymph node tissues

| <i>Mab</i> | <i>Source*</i> | <i>Dilution</i> | <i>Antigen</i> | <i>Reactivity</i> | <i>Reference</i> |
|------------|----------------|-----------------|----------------|-----------------------------|------------------------------|
| 17D | Basel | 5000 AF | CD4 | T-helper cells | Maddox <i>et al.</i> (1985) |
| SBU-T8† | Melbourne | 100 SN | CD8 | T-cytotoxic cells | Maddox <i>et al.</i> (1985) |
| 36F | Basel | 20 000 AF | CD2 | α - β T cells | Mackay <i>et al.</i> (1988) |
| CC15 | Compton | 100 000 AF | WC-1 | γ - δ T cells | Howard <i>et al.</i> (1989) |
| CC21 | Compton | 50 000 AF | CD21 | FDCs | Naessens & Howard (1991) |
| CC20 | Compton | 10 000 AF | CD1b | Mature B cells | Howard <i>et al.</i> (1993) |
| SBU-cII‡ | Melbourne | 256 000 AF | MHCII | Dendritic cells | Puri <i>et al.</i> (1987) |
| | | | | B cells | |
| | | | | Activated T cells | |
| | | | | Macrophages | |
| | | | | Dendritic cells | |
| VPM65 | Edinburgh | 5000 AF | CD14 | Monocytes | Gupta <i>et al.</i> (1996) |
| | | | | Macrophages | |
| 73B | Basel | 50 000 AF | CD45RA | B cells | Mackay <i>et al.</i> (1990) |
| | | | | T-cell subset | |
| VPM8 | Edinburgh | 200 SN | Ig LC | B cells | Yirrell <i>et al.</i> (1991) |
| VPM30 | Edinburgh | 800 SN | 28 kD | B cells | Naessens & Howard (1991) |
| | | | | Activated T cells | |

* Basel: Basel Institute for Immunology, Basel, Switzerland. Compton: BBSRC Institute for Animal Health, Compton Laboratory, Newbury, Berks, UK. Edinburgh: Dept Veterinary Pathology, University of Edinburgh, Edinburgh, UK. Melbourne: University of Melbourne, School of Veterinary Science, Parkville, Victoria, Australia.

CD, cluster designation; WC-1, workshop cluster 1; FDCs, follicular dendritic cells; Ig LC, immunoglobulin light chain; MHCII, major histocompatibility complex class II; AF, Mabs from ascitic fluid; SN, Mabs from tissue culture supernate.

† Clone 38-65.

‡ Clone 49.1.

salts fixative; ZSF). The present report describes the adaptation of this procedure for the demonstration of lymphocyte and other immune system cell markers in ovine tissues.

Materials and Methods

Tissue Sampling and Processing

Caudal, mediastinal, tracheobronchial and popliteal lymph nodes were obtained at necropsy from 10 sheep. Tissue slices 5–6 mm thick were immediately placed in a freshly prepared ZSF solution (0.1 M Tris base buffer with Ca acetate 0.05% [pH 7–7.4], containing Zn acetate 0.5% and Zn chloride 0.5%). The slices were fixed for 6–8 h at room temperature (RT) and then trimmed to 2–3 mm thickness before further fixation in ZSF for 24–72 h at RT. Tissue slices were then placed in plastic cassettes and transferred to 70% ethanol 30 min before processing to paraffin-wax by an automated procedure (80% ethanol, 45 min; 95% ethanol, 75 min; 99% ethanol, 3 × 75 min; isopropanol, 2 × 90 min; isopropanol/xylene [1:1 v:v], 90 min; xylene, 2 × 90 min; medium temperature melting [56–58°C] paraffin-wax, 2 × 105 min). Sections 5 µm thick were cut on a microtome, mounted

on treated glass slides (Superfrost Plus; Menzel-Glaser, Germany) and dried overnight at 37°C.

Immunohistochemistry (IHC)

Tissue sections were dewaxed and hydrated by conventional methods. Immunohistochemical labelling was performed manually with the Sequenza slide rack and coverplate system (Shandon, Runcorn, UK). Antibody binding was detected by the EnVision Plus HRP System (Dako, Ely, UK) which included two initial blocking steps, namely, (1) quenching of endogenous peroxidase activity with 0.03% hydrogen peroxide (included in the kit), for 5 min at RT, and (2) removal of non-specific tissue antigens by the application of 25% normal goat serum for 30 min at RT.

Monoclonal antibodies (Mabs) to several cluster designation (CD) or cell surface epitopes, at dilutions ranging from 1 in 25 to 1 in 800 for cell culture supernates and 1 in 1000 to 1 in 256 000 for ascitic fluids, were applied to sections. Two different incubation times (30 min and overnight) and temperatures (RT and 4°C) were used. Details of the antigenic epitopes sought, the cell subsets they define, the Mabs finally selected and their

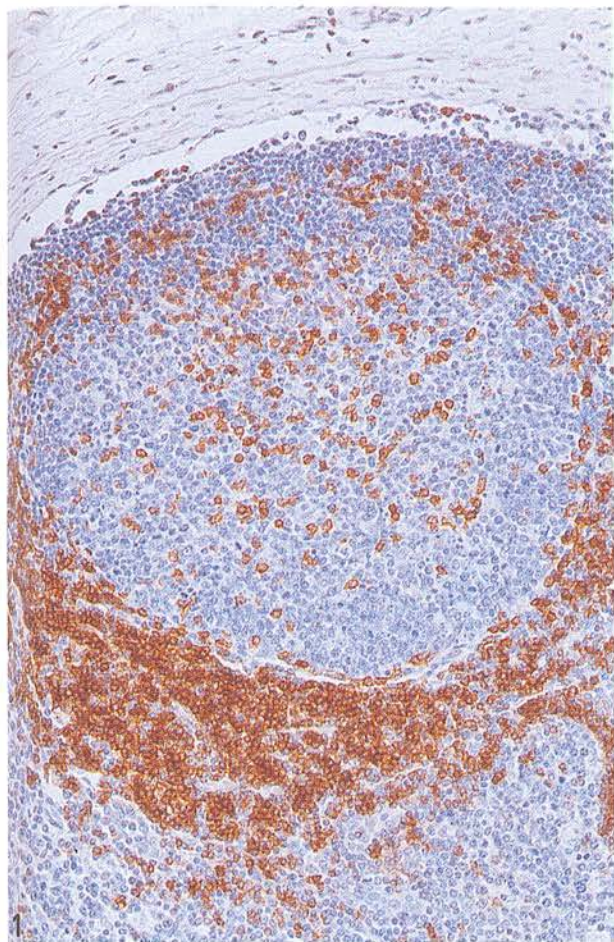


Fig. 1. Immunolabelling of CD4 epitopes by Mab 17D revealing grouped cells in the paracortex of the node and scattered cells inside the follicle. IHC and haematoxylin counterstain. $\times 164$.

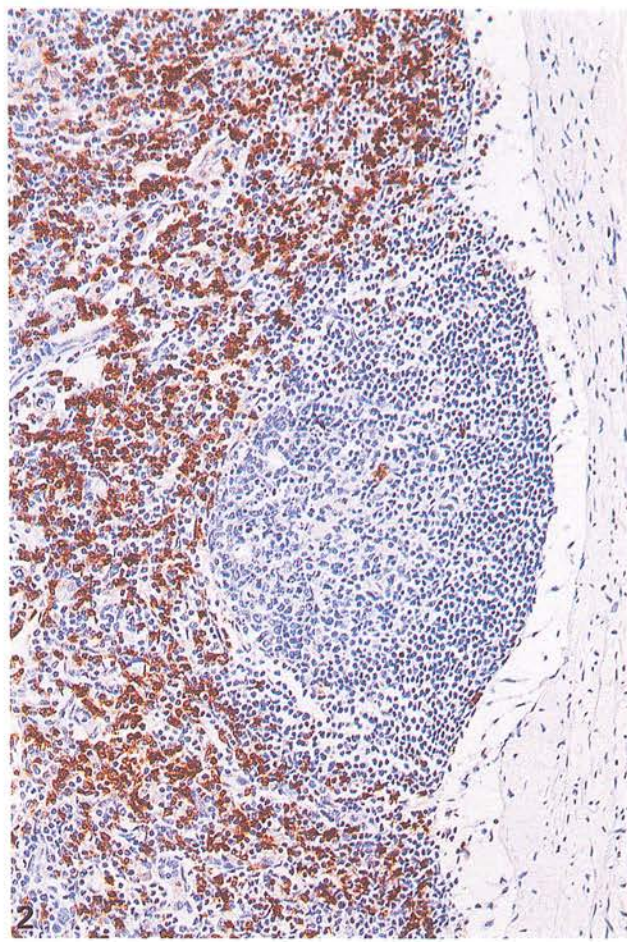


Fig. 2. Immunolabelling of CD8 epitopes with Mab SBU-T8 showing large groups of small lymphocytes in the paracortex of the lymph node. IHC and haematoxylin counterstain. $\times 164$.

source are given in Table 1. Other Mabs were also found to label the same epitopes, but to a lesser extent, and they are therefore omitted from this paper.

The secondary antibody (peroxidase-labelled polymer conjugated to goat anti-mouse immunoglobulins, included in the EnVision system kit) was applied for 30 min at RT to the tissue sections. Conjugate binding was detected by adding the substrate chromogen (3,3'-diaminobenzidine; DAB) and colour was allowed to develop for 7–8 min. Finally, tissue sections were washed with distilled water, removed from the assembly, counter-stained with haematoxylin, rinsed, dehydrated and mounted. Tris-buffered saline (TBS, 0.05 M Tris HCl, 0.15 M NaCl, pH 7.2–7.6) was used to wash tissue sections between each stage of the labelling procedure and to prepare normal goat serum and antibody dilutions. Omission of the

primary antibody was used to provide a negative control.

Results

The fixative and procedures (fixation, embedding and immunohistochemical) described above provided good preservation of histological detail. They also facilitated precise definition of the distribution of the various epitopes, including some regarded as sensitive to fixation or wax processing, or both. Optimal immunolabelling was produced by incubation with the primary antibody overnight at 4°C.

CD4 epitopes, detected with Mab 17D, were expressed by groups of small, round cells located in the paracortex of the lymph nodes immediately adjacent to the periphery of the follicles, as well as by some cells scattered inside the follicles (Fig. 1). Mab SBU-T8 labelled the CD8 epitopes on large

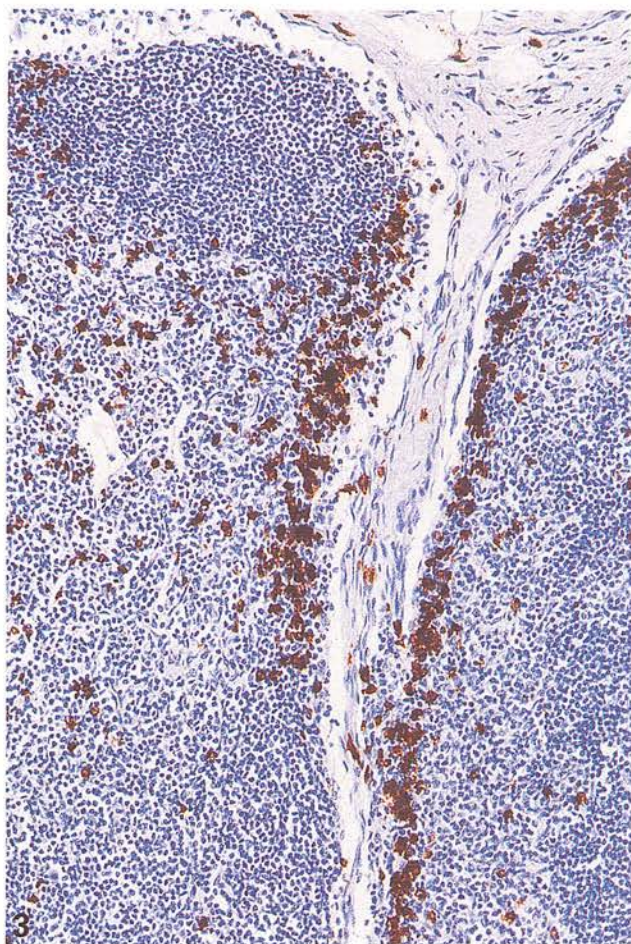


Fig. 3. WC-1 epitope labelling by Mab CC15. $\gamma\delta$ -T cells appear along the subcapsular sinus and scattered in the paracortex. IHC and haematoxylin counterstain. $\times 164$.

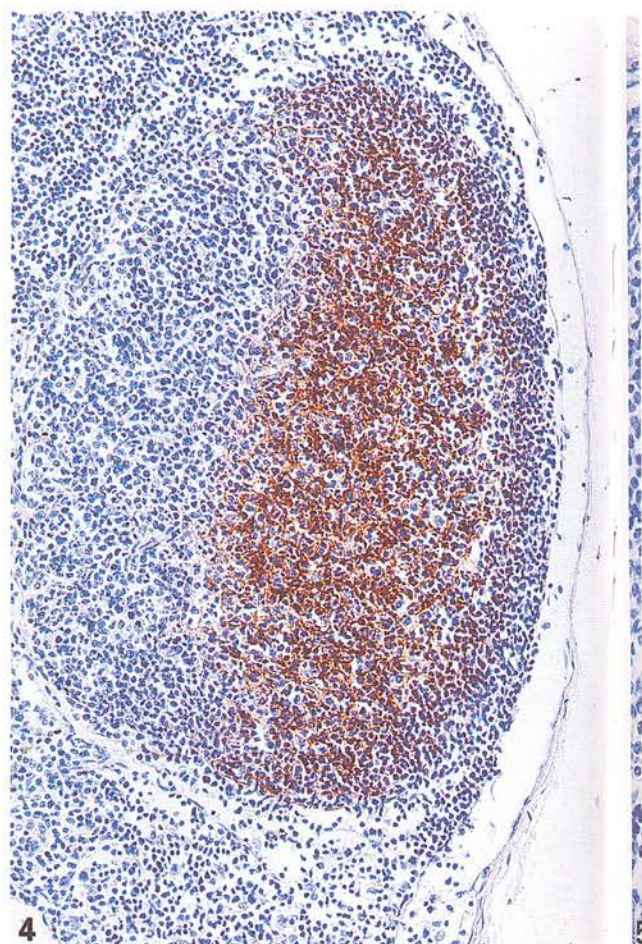


Fig. 4. CD21-positive follicular dendritic cells labelled with Mab CC21 in the germinal centre of the follicle. Note reticular pattern of labelling and restriction to the light zone. IHC and haematoxylin counterstain. $\times 164$.

populations of small round cells scattered or grouped in the paracortex of the nodes, whereas labelling was almost completely absent from the follicles (Fig. 2). CD2 epitopes were demonstrated with Mab 36F on large populations of grouped cells in the paracortex of the nodes and in a few scattered cells in the follicles. Their location corresponded to that of CD4⁺ and CD8⁺ cells, taken together. Immunolabelling of WC-1 molecules by Mab CC15 was present on scattered, relatively numerous, small round cells, mainly lining the subcapsular and trabecular sinuses but also scattered in the paracortex (Fig. 3).

Strong labelling of CD21 epitopes was present in the light zone of the germinal centres of lymphoid follicles when Mab CC21 was employed. Cells labelled by this antibody had a characteristic reticular or dendritic shape (Fig. 4). Mab CC20 labelled CD1b epitopes expressed by stellate cells, either scattered or in small groups, in the proximity

of the lymphoid follicles and often in the subcapsular sinus area (Fig. 5). MHCII antigens, identified with Mab SBU-cII, were demonstrated on large populations of small round cells within the cortical follicles and in the paracortex, as well as on scattered small cells in the medullary cords. At this last site, large cells with abundant cytoplasm lining the trabeculae were also labelled. The labelling was not as obviously localized on the cell membrane as with other markers, and had the appearance of being more intercellular (Fig. 6). Labelling of CD14 molecules by Mab VPM63 defined a population of large cells, with abundant cytoplasm and pale ovoid nuclei, that appeared either as scattered individual cells or as linear groups of cells in the medullary cords (Fig. 7). There was no labelling in the lymphoid follicles.

A restricted isoform of the common leucocyte antigen (CD45RA, 220 kDa) was identified with Mab 73B on large populations of small round cells

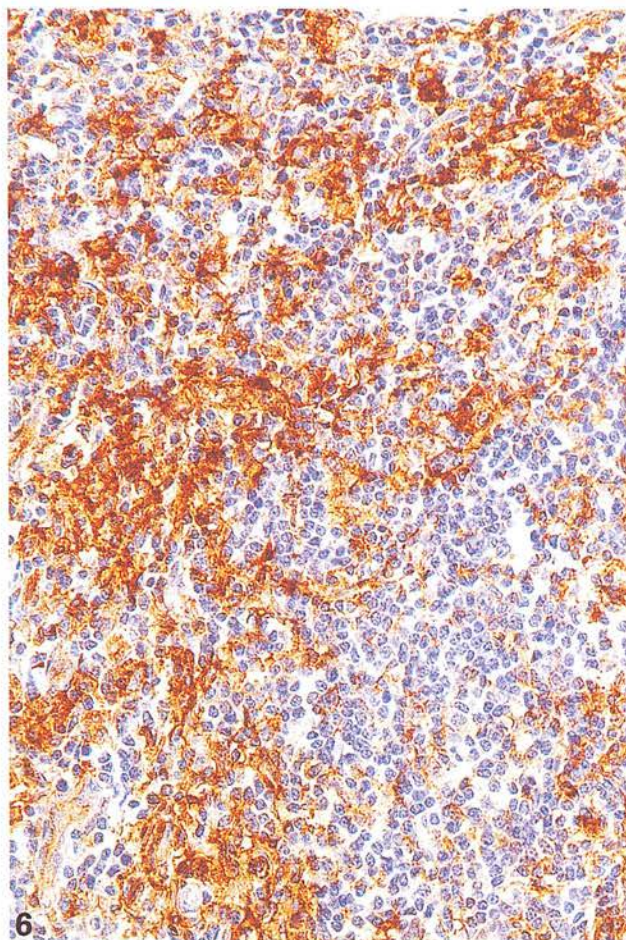
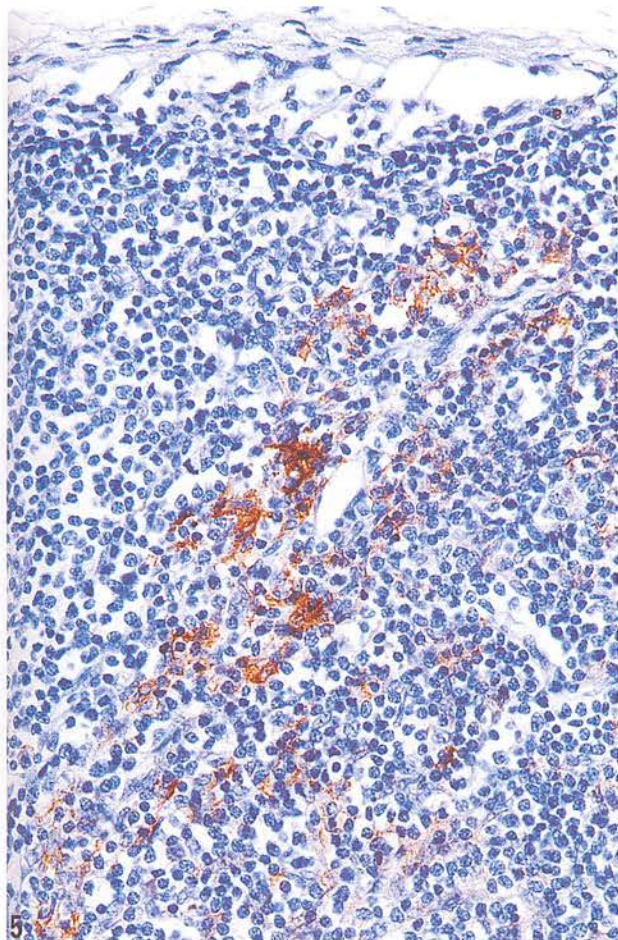


Fig. 5. CD1b-positive cells revealed by Mab CC20. The cells have a stellate or dendritic shape and are located in the subcapsular paracortex. IHC and haematoxylin counterstain. $\times 328$.

Fig. 6. Immunolabelling of MHC II antigens by Mab 49.1. Antigen is expressed in a slightly diffuse pattern by numerous cells in the paracortex of the node. IHC and haematoxylin counterstain. $\times 328$.

in the cortex of the lymph nodes, especially in the follicles but also in the paracortex. Mab VPM8, specific for the light chain of ovine immunoglobulins, resulted in strong cytoplasmic labelling of small scattered cells with abundant cytoplasm, located in the medullary cords and sinuses of the lymph node (Fig. 8), and also in light, cell-membrane labelling of some macrophage-like cells in the medullary cords. Labelling of a 28 kDa surface antigen with Mab VPM30 occurred on scattered cells with morphological and positional characteristics resembling those detected with Mab VPM8; it also occurred on small round cells scattered in the paracortex. With both VPM8 and VPM30 Mabs, there was an absence of labelled cells in the germinal centres of follicles.

Discussion

Cryo-preservation of tissue samples may provide optimal antigen preservation, but the preservation

of morphology is often sub-optimal, making precise location of the antigen-expressing cells within the tissue difficult or impossible. Light fixation of tissue samples (e.g., with paraformaldehyde or cacodylate-formalin) before cryo-preservation has been employed with good results, but in general, these methods have certain drawbacks, such as elaborate procedures for tissue handling and preliminary processing, and the need for ample storage space in appropriate freezers. It may also be difficult to produce cryostat sections of consistent quality and the handling of fresh tissues may pose biohazard problems. When aldehyde-fixation and paraffin wax-embedding are used, it is often necessary to apply antigen retrieval procedures before immunolabelling. Unfortunately, however, there is no "universal" antigen-retrieval procedure, but rather a wide choice of methods that have to be tried and if necessary adapted to suit the type of tissue or type of antigen. These methods are often laborious

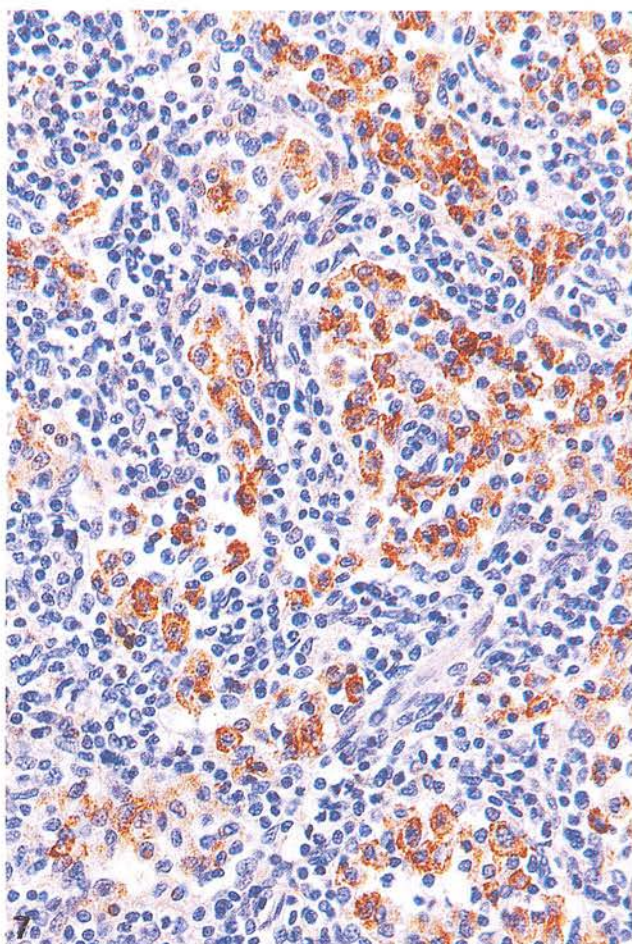


Fig. 7. Immunohistochemical demonstration of macrophages in the medullary sinuses of the lymph node. Mab VPM65 labels CD14-expressing cells, which have abundant cytoplasm and large pale nuclei. IHC and haematoxylin counterstain. $\times 328$.

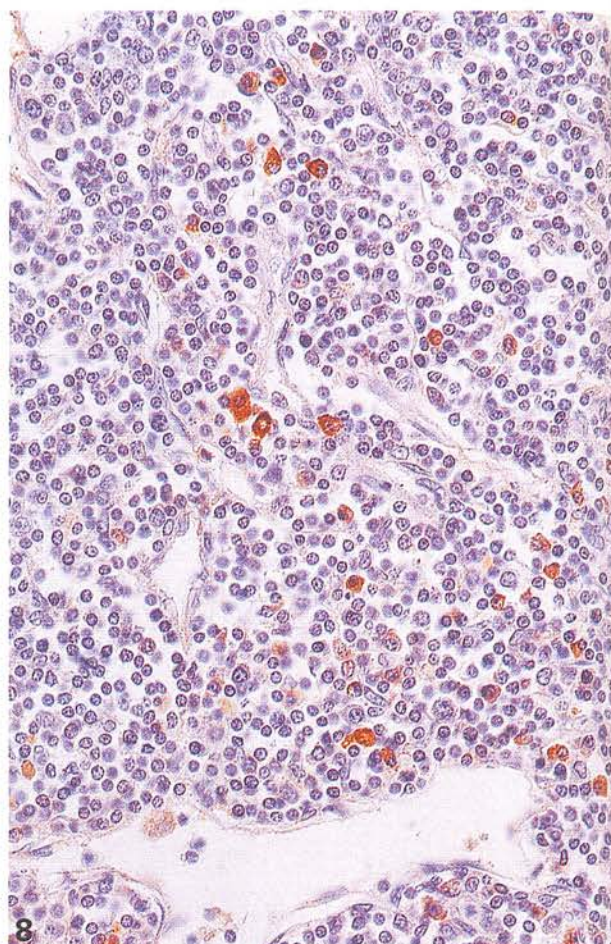


Fig. 8. Detection of immunoglobulins with Mab VPM8. Note the strong labelling in the cytoplasm of cells resembling plasmacytes located in the medullary cords. IHC and haematoxylin counterstain. $\times 328$.

and may require specialized equipment; they may also damage the tissue morphology (Cattoretti and Suurmeijer, 1995).

As far as the authors are aware, this is the first report of the use of ZSF, originally described by Beckstead (1994), for the preservation of ovine tissues. This method of fixation, followed by a routine paraffin wax-embedding procedure coupled with a highly sensitive method of immunolabelling, has made possible the demonstration of cell surface epitopes considered to be processing-sensitive, e.g., CD1, CD4 and CD8 (Beckstead, 1994). Preliminary data (not shown) of the performance of ZSF and standard fixatives, (e.g., formaldehyde and paraformaldehyde) confirmed this point and also indicated that the use of ZSF improved the immunolabelling of other more resistant epitopes, such as WC-1, MHC II, CD14 and CD21.

The results, based on immunolabelling and on the morphology, number and location of cells, accorded with previously reported reactivity or cell-specificity of the antigens under study (see references in Table 1). In this way, CD1, CD4 and CD8 reactivity would appear to represent dendritic cells, T-helper lymphocytes and T-cytotoxic lymphocytes, respectively. Moreover, detection of CD2, WC-1 and CD21 reactivity would appear to represent α - β T cells, γ - δ T cells and follicular dendritic cells, respectively. Some B cells might also show CD21 reactivity. CD45RA-positive cells would represent B cells and a subset of T cells. A wide range of cells within the lymph node (B cells, T cells, dendritic cells and macrophages) would express MHCII antigens. CD14-positive cells would correspond to macrophages (in this study, macrophages in the medullary cords, but not tinged body macrophages). Immunoglobulin-positive

cells would be regarded as plasma cells and occasionally macrophages, and 28 kDa-positive cells would be regarded as plasma cells and occasionally T cells. The failure to label B cells in the germinal centres of lymphoid follicles with VPM8 and VPM30 might be overcome by the use of other Mabs. Thus, for example, Mabs VPM13 (anti-IgM) and 2F1 (anti-IgE) have produced good results in other similar studies (Stanley *et al.*, personal communication).

The ZSF is inexpensive, easy to prepare and store, and of low toxicity (Beckstead, 1994). However, its major advantage is that it can be introduced as part of a set of routine procedures (tissue sampling, embedding in paraffin wax, cutting of sections and storing of blocks). In addition, ZSF gives good preservation of tissue morphology (almost comparable with that given by aldehyde fixatives) and does not require an antigen-retrieval step to permit adequate detection of cell surface epitopes. The identification of such markers will aid in the elucidation and evaluation of disease-induced pathological changes in animal tissues.

Acknowledgments

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Onset and Distribution of Tissue PrP Accumulation in Scrapie-affected Suffolk Sheep as Demonstrated by Sequential Necropsies and Tonsillar Biopsies

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Summary

Tonsillar biopsies (single or multiple) or necropsies, or both, were performed on sheep taken from a Suffolk flock in which frequent cases of scrapie had occurred over a period of several years. Clinically affected sheep of the susceptible PrP^{AQ/AQ} genotype had widespread disease-specific PrP accumulation in the central nervous system (CNS), lymphoreticular system and peripheral ganglia. In nine healthy PrP^{AQ/AQ} Suffolk sheep between 4 and 7 years of age, PrP could not be demonstrated *post mortem* in any of the lymphoreticular tissues, or in the peripheral ganglia or CNS. Tonsillar biopsies taken from animals of the resistant PrP^{AR/AR} and PrP^{AR/AQ} genotypes at age 3, 8, 14, 20 or 26 months did not show PrP accumulation. Disease-specific PrP accumulation in tonsillar biopsies from PrP^{AQ/AQ} sheep was not seen in 20 animals aged 3 months, but was found in two of 10 animals at age 8 months and in eight of 10 animals at age 20 months. The numbers of PrP-positive tonsillar biopsies obtained from sheep previously biopsied on more than one occasion was greater than the number of positive tonsils obtained from other susceptible sheep of comparable ages. The earliest disease-specific PrP accumulation seen was in tingible body macrophages within germinal centres and only later was it detected in cells resembling follicular dendritic cells. Fourteen PrP^{AQ/AQ} sheep examined *post mortem* at up to 17 months of age and which had not previously been biopsied or were biopsied only once had no CNS or tonsillar PrP accumulations. Two of these sheep subjected to necropsy at 14 months had PrP accumulation in lymphoreticular tissue, where it was confined to the mesenteric lymph nodes. In susceptible sheep, only low levels of immunohistochemically detectable PrP were present in a minority of follicles from tonsillar biopsies of young lambs, but by 14 months of age widespread PrP accumulation, affecting many or even all follicles, was present. Although clinical cases had widespread PrP accumulations in viscera, susceptible survivors had no such accumulations in tissues of the lymphoreticular system, peripheral nervous system or CNS, suggesting that some animals were not exposed to infection or were exposed to a non-infectious dose.

Introduction

Scrapie of sheep and goats is one of a group of slowly progressive neurological disorders known as the transmissible spongiform encephalopathies (TSEs), which are characterized by vacuolation of the central nervous system (CNS) and accumulation of an abnormal, partly protease-resistant form of

a host-coded cell surface sialoglycoprotein called prion protein (PrP^{res}). The detection of PrP in the brains of animals with TSE is important for confirming clinical diagnosis. However, the recently determined linkage between variant Creutzfeldt-Jakob disease and bovine spongiform encephalopathy (Bruce *et al.*, 1997) highlights the urgent need to develop a pre-clinical diagnostic aid



The potentially insecticidal *Narcissus pseudonarcissus* lectin demonstrates age-related mitogenicity

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Abstract

Lectins from monocotyledonous plants such as *Narcissus pseudonarcissus* (NPA) possess insecticidal properties and have the potential to increase pest resistance in transgenic crops. Therefore it is of interest to investigate the mitogenic properties of such lectins. Mononuclear cells purified from human umbilical cord and adult peripheral blood samples were stimulated with NPA and compared to phytohaemagglutinin as an example of a lectin from a dicotyledonous plant. Here we report that NPA is slightly mitogenic for adult human lymphocytes but mitogenicity is increased more than sevenfold for lymphocytes from umbilical cord blood. Similarly, NPA was found to be mitogenic for peripheral blood mononuclear cells (PBMC) from lambs and not adult sheep, supporting the age-related mitogenicity and indicating that further examination of the younger human population is warranted. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Microbiological Societies.

Keywords: Human lymphoproliferation; Mitogenicity; *Narcissus pseudonarcissus* lectin

1. Introduction

Plant lectins have been used as mitogens for several decades to evaluate immunocompetence. Most commercially available lectins are obtained from dicotyledonous plants, including phytohaemagglutinin (PHA) from the red kidney bean, *Phaseolus vulgaris*. An increased interest in monocotyledonous lectins has developed following the discovery that some lectins of the super-family Amaryllidaceae, such as snowdrop *Galanthus nivalis* (GNA) and daffodil *Narcissus pseudonarcissus* (NPA), possess insecticidal properties. Several prototype arable crop plants are now transgenic for GNA to enable preliminary assessment of their pest resistance [1,2]. Lectins have wide biological activity and concerns have been expressed about consumption of, or exposure to, crops transgenic for lectins. The few studies examining human subjects found GNA and NPA to be virtually non-mitogenic [3,4]. In one instance these preliminary reassuring observations were obtained with lymphocytes from adult donors, all over the age of

60 [5]. But we now report that the mitogenicity of NPA is age-related, with the lymphoproliferative response of the peripheral blood mononuclear cells (PBMC) in umbilical cord blood higher than in adult blood. Similarly, NPA was found to be mitogenic for PBMC from lambs and not adult sheep, supporting the age-related mitogenicity and indicating that further examination of the younger human population is warranted.

2. Materials and methods

2.1. Human subjects

In total, 12 human blood samples were collected, 7 umbilical cord bloods obtained during routine caesarean sections (kindly collected by Dr R. Hughes, Royal Infirmary of Edinburgh, following informed consent) and 5 volunteer adults, ages ranging from 24 to 56 years.

2.2. Sheep

In total, PBMC from 74 blood samples were assayed and analysed in three groups: lambs aged 4 days–1 month

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(Group A, $n=37$), 2–4 months (Group B, $n=26$), and adult sheep aged 1–3 years (Group 3, $n=11$). All animals were from the Moredun Research Institute.

2.3. Blood samples

Human umbilical cord and adult venous blood samples were collected into sterile tubes containing 10 units preservative free heparin per ml blood. The mononuclear cells were purified and adjusted to a final concentration of 10^6 cells ml^{-1} in RPMI-1640 medium (Life Technologies Ltd) containing antibiotics and 10% autologous plasma (human samples) or 10% inactivated foetal calf serum (sheep samples).

2.4. Cell culture with lectins

The optimum quantities, determined from a standard

curve, of commercially purchased PHA (Sigma, Poole, Dorset, UK) and NPA (Vector Laboratories, Peterborough, UK) were added to 10^6 PBMC ml^{-1} . Control samples (excluding lectin) were also prepared. All samples, at 2×10^5 cells/well were dispensed, (human samples five replicates, sheep samples in triplicate) into 96-well microtitre plates. The lymphocyte proliferation assays were performed in accordance with standard laboratory protocol [3] for a total of 96 h, with the addition of $0.7 \mu\text{Ci ml}^{-1}$ [^3H]thymidine (specific activity 2.0 Ci/mmol , Amersham, Little Chalfont, Buckinghamshire, UK) for the final 16 h. The cells were harvested and [^3H]thymidine uptake measured as cpm. For each sample the mean cpm of the replicates was calculated and the control mean cpm subtracted: S.E.M. was determined. Two-tailed student *t*-test determined the level of significance between the human adult and umbilical cord mononuclear cells, and between adult sheep and the two groups of lambs.

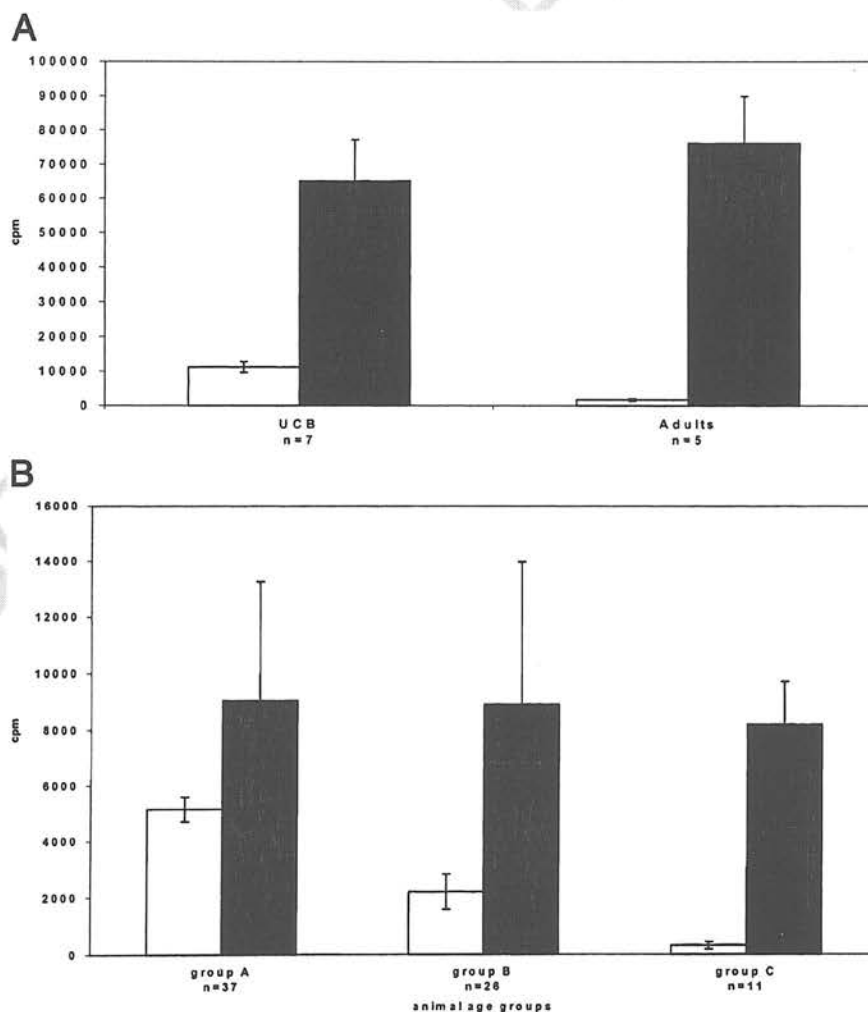


Fig. 1. In vitro lymphoproliferative response of PBMC to NPA (□) and PHA (■) stimulation. [^3H]thymidine uptake, shown as cpm \pm S.E.M. A: Human responses for umbilical cord blood (UCB) and adult blood. Comparison between the two groups was not statistically significant for PHA stimulation, but highly significant ($P=0.0004$) for NPA. B: Ovine responses for group A lambs aged 4 days–1 month, group B lambs aged 2–4 months and group C adult sheep aged 1–3 years. Comparison between the two groups of lambs (A, B) and the adults (group C) were not statistically significant for PHA stimulation, but were significant for NPA, at $P=0.0005$ for group A and $P=0.04$ for group B.

3. Results

PHA was strongly mitogenic for lymphocytes of both human age groups (umbilical cord mononuclear cells mean cpm $65\,058 \pm 12\,108$ and adult PBMC $76\,161 \pm 13\,770$) and there was no statistically significant difference in these responses (Fig. 1A). In contrast, the proliferative response to NPA stimulation was significantly higher ($P=0.0004$) with umbilical cord mononuclear cells (mean cpm $11\,322 \pm 1549$) compared to adult PBMC (mean cpm 1580 ± 413) (Fig. 1A).

In sheep, no statistically significant difference in the lymphocyte stimulation was found between the age groups in response to PHA (Fig. 1B). Stimulation with NPA gave a mean cpm of 5154 ± 445 and 2203 ± 616 for groups A and B respectively, compared to 330 ± 128 for the adult group C (Fig. 1B). The statistical significance of these results, in comparison to the adult group C, was calculated at $P=0.0005$ for group A and $P=0.04$ for group B.

4. Discussion

This study has provided clear evidence, in contrast to previous reports [4,5] that the monocotyledonous lectin, NPA, is mitogenic for human lymphocytes. We have confirmed that the proliferative response to NPA stimulation is age-related, with weak mitogenicity identified for adult human lymphocytes, and increasing more than sevenfold for lymphocytes from umbilical cord blood. Likewise, NPA was found to be mitogenic for PBMC from lambs and not adult sheep. As no age-dependent response to PHA stimulation was evident in either humans or sheep, and the lymphoproliferative responses to NPA in sheep reflected our observations in humans, the results presented

here suggest that age-related divergence in the mononuclear cell population affects the mitogenicity of NPA. Our findings relate only to in vitro studies and cannot predict in vivo responses to NPA. However, these results indicate that exposure of the human population to NPA could have physiological consequences. Therefore, to appreciate fully the potential implications of introducing foreign lectins into human and animal food sources, it is essential that we extend our knowledge of the plant lectin–mammalian cell relationship, not only for species specificity but to include all age groups.

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Systemic immune responses following infection with Jaagsiekte sheep retrovirus and in the terminal stages of ovine pulmonary adenocarcinoma

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Jaagsiekte sheep retrovirus (JSRV) is the aetiological agent of ovine pulmonary adenocarcinoma (OPA). To monitor changes in cellular immune function during JSRV infection, lymphoproliferation in response to various mitogens was measured in the blood of conventionally housed and specific-pathogen-free lambs experimentally infected with JSRV until the development of OPA and compared with uninfected control lambs. In addition, blood samples collected from adult field cases in the terminal stages of OPA and control adult sheep were compared. No difference in the proliferative response to phytohaemagglutinin and pokeweed mitogen between the animal groups was detected. In contrast, reduced responses to concanavalin A stimulation were demonstrated in the JSRV-inoculated lambs, prior to the onset of clinical disease, and also in the terminally ill adult sheep. Peripheral blood leukocytes were monitored to identify phenotypic frequency alterations. The CD4 lymphocytopenia and neutrophilia reported previously in adult OPA cases were demonstrated but similar phenotypic changes were not identified during experimental infection.

et al., 1998). The cell-mediated response to JSRV infection is, as yet, unknown, although CD4 lymphocytopenia and neutrophilia have been demonstrated in the peripheral blood of adult sheep during the terminal stages of OPA (Rosadio & Sharp, 1992; Holland *et al.*, 1999). A disseminated infection occurs as an early event, with viral RNA and proviral DNA found in cells of the immune system. Although very few cells are infected, the largest provirus load is in the monocytes/macrophages, followed by B cells, then T cells. However, there is no evidence of virus transformation of any of these cell types (Palmarini *et al.*, 1996b; Holland *et al.*, 1999).

The lack of appropriate antigens and JSRV-specific immunological assays has impeded investigations of immune responses during tumourigenesis in affected flocks. However, OPA can be induced experimentally in neonatal lambs by intratracheal inoculation with virus purified from tumour (Martin *et al.*, 1976), lung fluid (Sharp *et al.*, 1983) or infectious JSRV molecular clones (Palmarini *et al.*, 1999; DeMartini *et al.*, 2001). Thus, monitoring phenotypic and functional changes in the peripheral blood during experimentally induced tumourigenesis is possible.

The aim of this study was to investigate the systemic immune responses at all stages of JSRV infection, through tumourigenesis and into the terminal stages of OPA, and to establish if changes such as the CD4 lymphocytopenia and neutrophilia reported in adult OPA cases (Rosadio & Sharp, 1992; Holland *et al.*, 1999) were early events during JSRV infection, a consequence of tumourigenesis, or were demonstrated only in terminally ill animals. Moreover, the investigation was extended to identify alterations in cellular immune function attributable to JSRV infection.

The immune status in the terminal stages of OPA was determined by the lymphoproliferative responses to the mitogens phytohaemagglutinin (PHA), pokeweed mitogen (PWM) and concanavalin A (ConA) and phenotypic characteristics in a single blood sample collected from ten adult field cases (brought from various Scottish farms), compared with those of ten uninfected adult sheep (from stock at the Moredun

The β -retrovirus Jaagsiekte sheep retrovirus (JSRV) (Sharp *et al.*, 1983) is the confirmed aetiological agent of ovine pulmonary adenocarcinoma (OPA), known previously as sheep pulmonary adenomatosis and ovine pulmonary carcinoma (Palmarini *et al.*, 1996a, 1999). The immunological response to JSRV infection is poorly understood. No JSRV-specific humoral response has been detected (Sharp & Herring, 1983; Ortin *et*

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Research Institute). Systemic immune responses during the pre-clinical stages of disease were monitored in serial blood samples collected from six neonatal Dorset lambs inoculated intratracheally with JSRV purified from lung fluid, as described previously (Sharp *et al.*, 1983), and compared with four, age-matched, uninfected control lambs. To exclude the effects of external influences and to eliminate secondary infections, the investigation was extended to include specific-pathogen-free (SPF) lambs (six JSRV-inoculated and four control Suffolk-X lambs).

In all experiments, venous blood samples were collected into sterile, preservative-free, heparinized vacutainers (10 units heparin per ml of blood). Aliquots were removed for neutrophil counts. To adequately remove the large neutrophil population present in the blood of adult OPA cases, it was necessary to centrifuge the whole-blood sample for 15 min at 300 *g* prior to peripheral blood mononuclear cell (PBMC) purification using a standard laboratory protocol (Boyum, 1968). To maintain consistency, this procedure was performed with all of the blood samples. Purified PBMCs were adjusted to a final concentration of 10^6 cells/ml in RPMI 1640 medium (Gibco BRL) containing antibiotics and 10% inactivated foetal calf serum. Plasma was collected also for the detection of JSRV capsid (CA)-specific antibodies.

When symptoms of OPA were apparent, the animals were humanely killed by an overdose of pentobarbitone (Rhone Merieux) and necropsy was performed. Diagnosis of OPA, based on the characteristic gross and histopathological lesions described elsewhere (Sharp & Angus, 1990), confirmed the disease status of the experimentally infected lambs and adult sheep. For the conventionally housed lambs, OPA was confirmed in three lambs at 12 weeks, one lamb at 16 weeks and one lamb at 20 weeks of age. In the SPF experiment, OPA was confirmed in one lamb at 9 weeks, one lamb at 18 weeks and one lamb at 20 weeks of age. In each experiment, two control lambs were culled at 16 weeks and the remaining two control lambs were culled at 20 weeks; none of the lambs showed lesions of OPA.

To demonstrate that phenotypic frequency alterations occur in adult OPA cases but not during experimental JSRV infection, PBMC phenotyping by indirect immunofluorescence was performed using standard laboratory procedures (Bachh *et al.*, 1995; Lloyd *et al.*, 1995). Cells were immunolabelled with a panel of mouse anti-sheep monoclonal antibodies specific for CD2 T cells (I35A), CD4 T cells (17D), CD8 T cells (SBU-T8), $\gamma\delta$ T cells (86D), B cells (α -light chain VPM8) and MHC class II antigens (VPM54); all antibodies were supplied by Professor John Hopkins, University of Edinburgh, Edinburgh, UK). The secondary antibody was an FITC-conjugated rabbit anti-mouse immunoglobulin (DAKO) and the results were determined by flow cytometry (EPICS XL-MCL Coulter Electronics). The percentage of neutrophils present in the white blood cell population was determined by flow cytometry based on cell size and granularity, following red blood cell lysis with

Erthyrolyse (Serotec). The mean percentage for each animal group was determined \pm SEM. Student's two-tailed *t*-test determined the level of significance between the groups.

In adult sheep with OPA, a significant decrease ($P = 0.02$) in the percentage of positively immunolabelled CD2 T cells ($42 \pm 3\%$) was detected in the blood compared with uninfected controls ($52 \pm 3\%$). The depletion in the T cell population was further characterized by a CD4 lymphocytopenia, with a significantly reduced ($P = 0.004$) percentage of CD4 T cells ($16 \pm 2\%$) detected in the adult OPA cases compared with the control sheep ($24 \pm 2\%$). No significant frequency alterations were identified for CD8 T cells, $\gamma\delta$ T cells, B cells or MHC class II antigens. The percentage of neutrophils present in the blood showed a significant increase ($P = 0.05$) in the terminally ill adult OPA cases ($52 \pm 6\%$) compared with the control sheep ($33 \pm 6\%$). In contrast, no statistically significant phenotypic frequency alterations were identified, at any time during the 20 weeks after inoculation, in the blood of the experimentally JSRV-infected lambs (conventionally housed and SPF) in comparison with their respective controls.

To show that ConA lymphoproliferative responses are reduced in experimentally JSRV-infected lambs and adult sheep with OPA, the optimum concentration for each mitogen, 12 μ g/ml for PHA, 15 μ g/ml for ConA and 2 μ g/ml for PWM (Sigma), was added to 10^6 PBMCs per ml RPMI 1640 medium. Control samples (excluding lectin) were also prepared. Lymphoproliferation was performed at 2×10^5 PBMCs per well (in triplicate) in accordance with standard laboratory protocols (Kilpatrick, 1998) for a total of 96 h, with the addition of 0.7 μ Ci per well [*methyl*- 3 H]thymidine (sp. act. 2.0 Ci/mmol, Amersham) for the final 16 h. [3 H]Thymidine uptake was measured as c.p.m. For each sample, the mean c.p.m. of the replicates was calculated and the control mean c.p.m. subtracted. The resulting individual values were used to calculate the mean c.p.m. of each animal group \pm SEM. Student's two-tailed *t*-test determined the significance of the differences between the mean c.p.m. of the different groups.

No significant difference in the level of response to PHA stimulation was identified between the adult OPA sheep and the controls. In contrast, a significantly reduced response ($P = 0.03$) to ConA stimulation was evident in the PBMCs of adult OPA cases (10955 ± 2532 c.p.m.), which was only 58% of the response observed in the controls (19000 ± 2426 c.p.m.). No difference in response to PHA (Fig. 1a) or PWM (Fig. 1b) stimulation was detected between JSRV-inoculated and conventionally housed control lambs. However, the infected lambs were significantly ($P = 0.002$ – 0.008) less responsive to ConA stimulation than the control animals (Fig. 1c). This observation was detected first when the lambs were 8 weeks old; at that time, the response in the JSRV-infected group was only 33% of that in the control lambs. Reduced responses to ConA stimulation in excess of 75% were demonstrated in the infected lambs until the end of the experiment at 20 weeks. The lymphoproliferative responses of the infected SPF lambs also

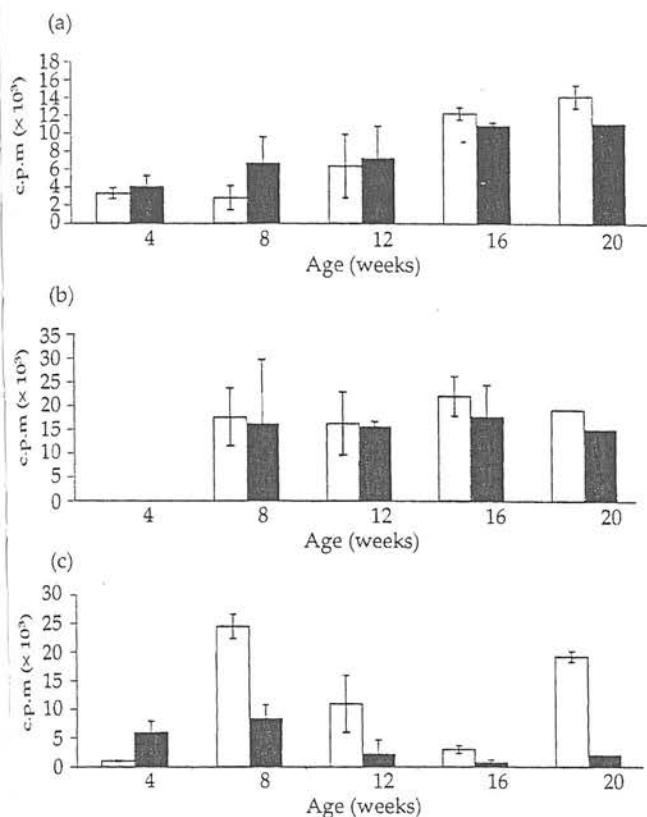


Fig. 1. Lymphoproliferative response to PHA, PWM and ConA stimulation during experimental JSRV-induced OPA. PBMCs purified from blood samples of conventionally housed JSRV-infected and control lambs were stimulated with PHA, PWM and ConA. Histogram bars represent group means \pm SEM for each mitogen. [3 H]Thymidine uptake is shown as c.p.m. (a) Response to PHA; (b) response to PWM; (c) response to ConA. Statistical significance, $P = 0.002-0.008$. Control lambs (open bars) were tested at 4, 8, 12, 16 ($n = 4$) and 20 ($n = 2$) weeks of age. JSRV-infected lambs (closed bars) were tested at 4, 8, 12 ($n = 6$), 16 ($n = 3$) and 20 ($n = 1$) weeks of age.

demonstrated a reduced response to ConA stimulation (results not shown).

JSRV CA-specific antibodies were not detected by indirect sandwich ELISA. Plasma collected from all the sheep were tested for the presence of JSRV CA antibodies by ELISA. A recombinant JSRV CA-coating antigen was prepared by cloning part of the JSRV *gag* gene (nucleotide sequence published by York *et al.*, 1991) into the plasmid pBAD/His A (Invitrogen) and expressed in *Escherichia coli* LMG 194 cells. The plasmid without the CA insert was used as a negative control-coating antigen. Sheep anti-CA serum, as a positive control, was generated according to Palmarini *et al.* (1995) with some minor adaptations. Specific absorption values were calculated by subtracting the control values from the test values. The cut-off point to define seropositivity was set as the mean A_{450} of a negative control serum (pre-immunization serum) plus three times the standard deviation of the mean (four replicates); none of the plasma samples from the

experimental lambs or adult sheep with OPA contained any CA antibodies.

The present investigation of lymphoproliferative responses to mitogen stimulation has revealed for the first time an alteration in host cellular response due to the presence of JSRV. A reduced response to ConA stimulation was evident in the PBMCs of experimentally JSRV-infected lambs as an early event during tumourigenesis, being apparent from 8 weeks of age, prior to clinical disease. Moreover, this functional alteration was evident in the adult OPA sheep tested.

There are many examples of retrovirus-encoded proteins with immunosuppressive potential but, during JSRV infection, there has been no evidence of virus transformation of immune cells, although a disseminated infection is evident (Holland *et al.*, 1999). Despite the low virus load, the results of this study show that the presence of JSRV does alter cellular function. During the early stages of human immunodeficiency virus (HIV) infection, when only a small percentage of CD4 lymphocytes are infected, the cellular function of the T helper cell population is adversely affected prior to their depletion (Miedema *et al.*, 1988). It has been established that retrovirus suppression can be selective for one mitogen without the response to other mitogens being affected. For example, HIV-1 inhibits PHA-induced lymphoproliferation but does not affect the response to ConA stimulation (Mann *et al.*, 1987). The transmembrane protein p15E from murine leukaemia virus (Ruegg *et al.*, 1989; Schmidt *et al.*, 1987) and feline leukaemia virus (Mathes *et al.*, 1978, 1979) induces immunosuppression by preventing aggregation of ConA receptors, suggesting that expression of this protein interferes with the redistribution of the cytoskeleton (Dunlap *et al.*, 1979). Therefore, although immune cells may not be transformed by JSRV, specific changes in glycoprotein-bound carbohydrate residues during JSRV infection may be involved.

If no JSRV-specific adaptation at the cell surface occurs, then virus modification of post-recognition events, such as transport mechanisms and the intracellular pathways utilized during mitogenesis, may be implicated. Recent studies have identified that the cytoplasmic moiety of the JSRV envelope is sufficient to transform rat and mouse NIH 3T3 cells (Maeda *et al.*, 2001; Rai *et al.*, 2001). Phosphorylation of protein kinase B was detected in all JSRV-transformed cells, which would indicate that expression of the JSRV envelope activates the phosphatidylinositol 3-kinase (PI-3K) pathway (Palmarini *et al.*, 2001). PI-3K phosphorylation of protein kinase B is known to inhibit apoptotic mechanisms and is essential for the transition from G_1 -to-S phase during the lymphoproliferative response to several mitogens (Roche *et al.*, 1994). Therefore, signal transduction and regulatory mechanisms may be influential by contributing not only to the neoplastic process but also in determining the altered cellular response of JSRV-infected sheep.

Our knowledge of the PBMC phenotypic characteristics already identified in OPA cases was extended during this

present study. No evidence of the CD4 lymphocytopaenia and neutrophilia, detected in the blood of adult sheep during the terminal stages of OPA (Rosadio & Sharp, 1992; Holland *et al.*, 1999) and confirmed in this study, was revealed during experimental disease. This may suggest that these changes do not occur in direct response to JSRV infection but are a consequence of increased susceptibility to secondary infections encountered as the health of the animal declines.

In adult OPA cases, no evidence of circulating JSRV-specific antibodies has been recorded either by Western blotting or by ELISA (Sharp & Herring, 1983; Ortin *et al.*, 1998). In addition, this study demonstrated a lack of specific antibodies for the JSRV CA during acute experimental JSRV infection and OPA development.

With no circulating JSRV-specific antibodies detected and phenotypic alterations only demonstrated in adult OPA cases, the reduced response to ConA stimulation indicates an alteration in systemic immunity. This change is an early event during JSRV infection, sustained through tumourigenesis and into the terminal stages of OPA.

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